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13. ABSTRACT *(Maximum 200 Words)*

Yeast two-hybrid screens for proteins interacting with the integrin linked kinase, ILK1, identified a protein phosphatase 2C, ILKAP, that blocks ILK1 activity and signalling. A second interacting protein contains two calponin homology (CH) domains, suggestive of actin-binding function. Conditional expression of ILKAP indicated that it selectively inhibited ILK1 kinase activity, and subsewuent signalling through GSK3ß and ß-catenin, components of the Wnt signal transduction pathway. This down-regulation requires ILKAP catlytic activity. A second interacting protein, Clint, contains a tendem arrangement of two calponin homology (CH) domain suggestive of actin-binding function. ILK1 binds to the C-terminal CH domain of Clint. Clint mRNA is highly prferentially expressed in human straited muscle tissues, and an apparently single protein of apparent molecular weight of 35 kDa is recognized by antibodies raised to a Clint fusion protein. Interaction of ILK1 with Clint may require the catalytic function of ILK since a kinase-dead version of ILK was deficient for Clint binding. As this kinase dead ILK1 acts as a dominant negative mutation, this suggests Clint interactions are important in ILK1-mediated signalling. ILK1 can phosphorylate recobinaint Clint protien, indicating that Clint could be a physiologic substrate for ILK1. We characterize a regulator and a potential substrate, of ILK1.

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1. INTRODUCTION

The Integrin linked kinase (ILK) is a potent transforming oncogene in cultured epithelial cells (Hannigan et al., 1996). Recent work has implicated ILK activation in several major human cancers. ILK protein and activity levels are markedly elevated in colonic polyps of familial adenomatous polyposis (FAP) patients, indicating an early role in development of colon cancer (Marotta et al., 2001). Similarly, increased ILK expression and kinase activity correlate positively with malignant progression of human prostate carcinoma (Graff et al., 2001). Indeed, targetted expression of ILK in the mammary epithelium of transgenic mice induces invasive breast adenocarcinomas (White et al., 2001). A number of studies have shown that ILK can regulate glycogen synthase kinase 3-ß (GSK3ß) and protein kinase B (PKB) in tissue culture (Delcommenne et al., 1998; Persad et al., 2000; Persad et al., 2001; Wang et al., 2001), suggesting ILK transforms cells through one or more pathways involving these two important regulators of cell division, differentiation and apoptosis. While the importance of ILK oncogenic signalling is clear, little is known of how this conserved kinase is negatively regulated. Using a yeast two-hybrid screen to identify potential regulators of ILK we have discovered a highly conserved protein phosphatase, ILKAP, that binds to ILK and inhibits its signalling (Appendix 1, and see Figure 2). Most strikingly, ILKAP phosphatase activity selectively regulates ILK signalling through GSK3ß, thereby potently inhibiting the ß-catenin oncogenic pathway (Leung-Hagesteijn et al., 2001).

1.1 - ILK signalling is dysregulated in cancer

Integrin Linked Kinase, ILK, was discovered in a screen to identify intracellular signal transduction molecules that associate with ß1 integrins (Hannigan et al., 1996).

Genetic proof of the functional association of ILK and ß-integrins has subsequently come from studies in *Drosophila* (Zervas et al., 2001). The discovery of ILK provided a critical insight to the understanding of how ß1 integrins transduce extracellular signals to effect cell regulation. We first described the physical association of ILK with integrin subunits in yeast two-hybrid and co-precipitation analyses, and showed that ILK overexpression in epithelial cells induced anchorage independent growth and tumourigenesis in nude mice (Hannigan et al., 1996; Radeva et al., 1997; Somasiri et al., 2001). We demonstrated reduced cell adhesion to extracellular matrix proteins in ILK over-expressing cells, providing a functional correlate of the physical association between ILK and integrins (Hannigan et al., 1996). In mammalian cells ILK activity is transiently stimulated through occupation of integrins by extracellular matrix (ECM) proteins such as fibronectin and collagens, and is a major regulator of integrin signal transduction. Since its initial discovery ILK has been shown to have more generalized signalling properties important for regulation of cell growth, differentiation and survival, in response not only to integrin engagement, but also to activated receptor tyrosine kinases such as those for hepatocyte growth factor and insulin-like growth factors (Dedhar et al., 1999).

ILK is a potent regulator of ß-catenin activation (Novak et al., 1998), a common oncogenic event in human tumours, including colon, prostate and liver carcinomas (Polakis, 1999; Polakis et al., 1999). In addition, ILK activation requires the activity of phosphoinositide 3'-OH kinase (PI3K), a inositol lipid kinase active in vital cellular processes such as signal transduction, apoptosis, differentiation, and membrane trafficking. Dysregulation of PI3K signalling is a common feature in the development of prostate and breast carcinomas, as well as brain tumours such as glioblastoma through loss of PTEN phosphatase activity (Simpson and Parsons, 2001; Teng et al., 1997; Tsou et al., 1997). PI3K activity is antagonized by the PTEN/MMAC lipid phosphatase, encoded by a tumour suppressor gene on chromosome 10q23 that is mutationally inactivated in a large number of sporadic, and also some hereditary cancers. ILK activity is elevated in cells overexpressing the p110 catalytic PI3K subunit, or in PTEN-/- prostate carcinoma cells, and reintroduction of PTEN cDNA to these latter cells restores ILK activity to control levels (Persad et al., 2000). Thus, ILK activity is stimulated by

genetic loss of a tumour suppressor, and in turn hyperactivates the ß-catenin oncogenic pathway (Figure 1), implicating dysregulated ILK signalling in a variety of cancers.

1.2 - Oncogenic targets of ILK signalling

An important cellular target of ILK is the serine/threonine protein kinase B (PKB, cellular homologue of the v-Akt oncogene), a much-studied intracellular regulator of mitogenesis and cell survival. Two distinct phospholipid-dependent kinase activities, PDK1 and PDK2, are required for full activation via phosphorylation of PKB at Thr 308 and Ser473, respectively (Alessi and Cohen, 1998; Alessi et al., 1997) Overexpression of ILK in Scp2 mammary epithelial cells activates PKB and inhibits anoikis (suspension-mediated apoptosis), likely involving regulation of the activities of caspases 3 and 8 (Attwell et al., 2000). Stimulation of ILK activity by IGF1 or ECM induces membrane translocation and activation of PKB in intact epithelial cells, the latter via phosphorylation at Ser473. Moreover, recombinant ILK phosphorylates PKB at Ser473 *in vitro*, supporting the notion that PKB is a physiologic substrate of ILK (Delcommenne et al., 1998; Persad et al., 2000; Persad et al., 2001). The PH domain of PKB is responsible for directing some aspects of its membrane localization and activation, however the PDK1 PH domain is necessary and sufficient for insulin-stimulated membrane translocation of associated PKB (Currie et al., 1999; Filippa et al., 2000). The ILK PH domain is thought to be required for interactions of integrin-localized ILK with activating lipids (Delcommenne et al., 1998). Thus, a number of studies have suggested that ILK is a PDK2, regulating PKB-dependent cell division and apoptosis.

Genes encoding key proteins of the Wnt pathway are common targets of oncogenic mutation (Peifer and Polakis, 2000; Polakis, 2000). Of these, that for ß-catenin is one of the most frequently altered (Barker and Clevers, 2000; Morin, 1999; Polakis, 1999). In the normal case, Wnt stimulation of cells leads to inhibition of GSK3ß (Ferkey and Kimelman, 2000; Kim and Kimmel, 2000), which serves to stabilize ß-catenin, resulting in its nuclear accumulation and interaction with Tcf/Lef transcription factors (Figure 1). Known gene targets of ß-catenin/Lef transactivation include c-Myc, and also encode cell cycle regulators and matrix remodelling proteins, among others (Brabletz et al., 2000; He et al., 1998). GSK3ß activity phosphorylates ß-catenin and targets if for proteasomal

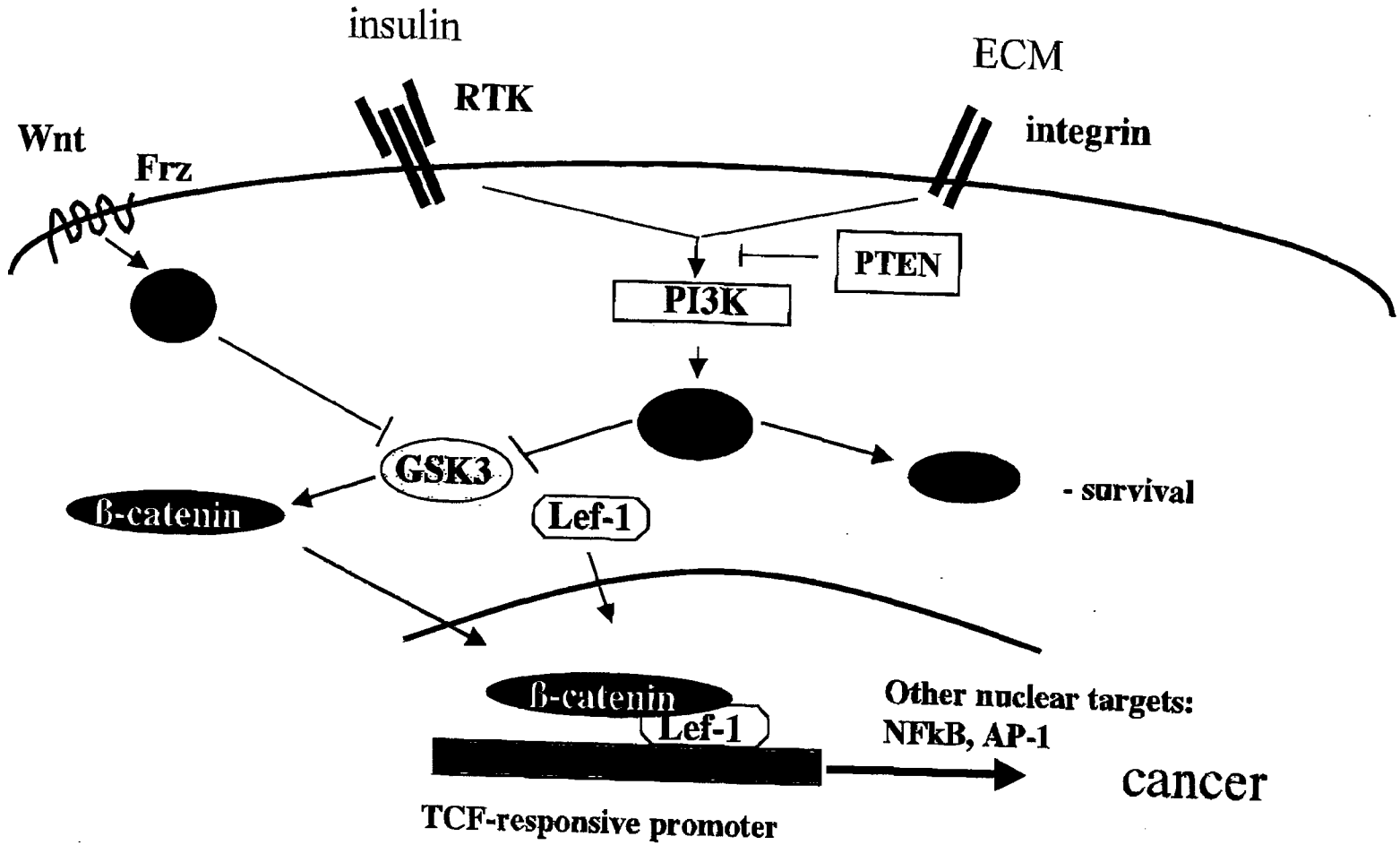

Figure 1: Oncogenic signalling by ILK. Stimulation of ILK leads to activation of PKB and inhibition of GSK3ß activities, resulting in stabilization of ß-catenin. S-catenin translocates and associates with Lef transcription factors to activate target genes such as c-myc. dysregulation of Ilk signalling via loss of PTEN tumour suppressor function leads to constitutive activation of the ß-catenin oncogenic pathway. Wnts interact with Frizzled receptors (Frz) to inhibit GSK3 activity, through the effector Dishevelled (Dsh). It's not known if Wnt-mediated inhibition of GSK3 involves ILK.

degradation (Aberle et al., 1997; Barker and Clevers, 2000), thereby maintaining a low level of ß-catenin/Lef target gene transcription. Similarly, active but not kinase-deficient ILK inhibits GSK3ß activity and stabilizes ß-catenin, suggesting ILK is an important regulator of GSK3 signalling (Delcommenne et al., 1998; Troussard et al., 2000). In addition, cell cycle regulation by ILK involves GSK3ß-dependent transactivation of the cyclinD1 promoter (D'Amico et al., 2000), thus stimulation of cyclin D1/Cdk4 activity represents another mechanism of ILK-mediated oncogenesis.

2. OVERVIEW OF PROGRESS

2.1 Regulation of ILK1 activity by ILKAP, a new protein phosphatase 2C

A major focus this work has been characterization of the ILK1 signal transduction pathway and we have made significant progress toward this end, by identifying two new protein partners of ILK1. The first of these, ILKAP, is an ILK1-associated phosphatase that selectively inhibits ILK activity, and which functions to down-regulate ILK1 signal transduction. ILK1 has been shown to stimulate the Wnt developmental signal transduction pathway, and we have found that ILKAP selectively inhibits ILK1-stimulated signalling of the Wnt pathway. Specifically, ILKAP expression in HEK 293 epithelial cells inhibits ILK1-induced phosphorylation of glycogen synthase kinase 3, GSK3, a critical regulator of the Wnt signalling cascade. Details of the ILKAP isolation and characterization are found in Appendix I (Leung-Hagesteijn et al., 2001). As well, non-published data on ILKAP, accrued during the period of isolation and characterization were included in previous annual reports for DAMD17-97-1-7092.

2.2 CLINT, an ILK1 binding homologue of C. elegans muscle development gene

A second ILK1-interacting protein, CLINT, appears to be a target of regulation by ILK1. CLINT is a calponin homology domain-containing protein that is preferentially expressed in striated muscle. In vitro assays indicate that CLINT can serve as a substrate of ILK1, suggesting a regulatory interaction. Recently it has been shown that a *C. elegans* homologue of CLINT, pat-6, co-localizes with the ILK1 homologue, pat-4, in myofibrillar lattice structures of developing muscle cells (Hresko et al., 1994; Williams and Waterston, 1994). This suggests that the conserved interaction between ILK1 and CLINT is important in vertebrate muscle development. We have established a

collaboration with Dr. B. Williams at U. Illinois, to pursue genetic analyses of CLINT mutants generated in our lab, as a complement to the molecular and biochemical characterization done in my lab. I do not have any data from this collaboration in my possession, as yet.

2.3 ILK1 is a critical mediator of in vitro myoblast differentiation

My lab has recently shown that ILK1 is required for in vitro differentiation of L6 myoblasts to myotubes (Appendix II, Miller et al., J. Biol. Chem., in revision), providing us a model biological system in which to study the ILK1 signal transduction pathway. We show very clearly that expression of a dominant negative, kinase inactive variant of ILK1 blocks L6 myoblast differentiation, at both the biochemical and morphological levels. Although ILK1 has been shown to activate protein kinase B (PKB, cellular homologue of v-Akt oncogene), we do not see significant inhibition of PKB activation under ILK1-inhibited conditions. Therefore, although ILK1 is inhibited in the ILK dominant negative myoblasts, the PKB survival pathway appears intact. Our work with ILKAP in 293 cells suggests the Wnt developmental pathway could be an important target of ILK1 signalling in differentiating myoblasts.

3. CLINT CHARACTERIZATION

3.1 Identification of CLINT in yeast two-hybrid screens for ILK interacting proteins

We isolated a 700 bp cDNA from a yeast two-hybrid interaction trap screen, which has been described in my previous annual reports. This cDNA, which we designated 3B7, was sequenced on both strands and found to contain a 450bp open reading frame, coding for 149 amino acids and a poly A tail 245bp downstream of the stop codon, although no polyadenylation site (AATAAA) was apparent. BLAST homology searches indicated the 149 amino acid 3B7 peptide represented a novel human protein, which was conserved in the sequence of a putative *C. elegans* cytoskeletal protein (Accession Number AF016687). The 3B7 amino acid sequence showed 56% identity to the *C. elegans* α-actinin-like protein which was identified by the GENEFINDER program, from the genomic sequence of *C. elegans* chromosome 3. This report will summarize our characterization of the protein represented by the 3B7 isolate.

3.2 Characterization of CLINT cDNA

In order to isolate a longer cDNA clone, a variety of strategies were used. First, the 3B7 cDNA was radioactively labelled with (Buyon et al., 1997) and used to screen numerous libraries which included placental phage library, muscle phage library, and a human heart bacterial library. In addition, two Origene Rapid Screen cDNA libraries (placental and skeletal muscle) were screened by PCR using a 3B7 specific 3' primer and a 5' vector primer. Using NCBI BLAST server, Expressed Sequence Tags (ESTs) were identified which overlapped with 3B7. Three EST IMAGE clones (74203, 1675981, 625520), which aligned with 3B7 cDNA sequence, were ordered and sequenced. The majority of positive clones (2B, 3B, 1675981, 74203) isolated from these various sources only yielded sequence spanning the 3B7 cDNA. Although most clones contained a poly A tail at the same location as 3B7, one clone (3B) was isolated which extended the 3B7 cDNA 300bp in the 3' direction before ending in a poly A tail. The sequence of clone 3B contains a polyadenylation signal 17bp upstream of the poly A tail. Closer inspection of the ESTs which aligned with 3B7 revealed that many contained the extended 3' untranslated region (UTR) sequence found in 3B. ESTs with the shorter 3' UTR sequence were also present. Although these libraries were oligo dT primed it is possible that the two 3'UTR are a result of alternative splicing. Genomic sequence analysis showed that the entire 3'UTR, including the addition 300bp, is coded by a single exon therefore it is unlikely that the differences in the 3' UTR of 3B7 are due to alternative splicing. Since this difference in the 3'UTR sequence is consistent for isolates from independent sources we speculate that the two poly A tails arise due to different polyadenylation sites.

Due to the difficulty in obtaining full length clones from cDNA libraries a different approach was taken. BLAST homology searches using 3B7 cDNA identified a 1347 bp cDNA in the database, CGI-56 (Accession Number AF151814). CGI-56 was isolated by PCR using degenerative primers to the *C. elegans'* α-actinin-like cDNA (Acession Number AF016687) in the search for a novel human gene. Primers based on the 5' end of CGI-56 and the 3' end of 3B7 were used in RT-PCR of human

rhabdomyosarcoma cell RNA. The two PCR reactions (using two different 5' primers) produced 2 single bands of expected sizes, 1.1kb and 1.3kb, based on the CGI-56 cDNA. These PCR products were cloned and sequenced on both strands.

3.3 Chromosomal localization of CLINT gene.

FISH localized CLINT to a single chromosome however it is possible that there are genes in tandem that code for CLINT. Recently the entire chromosome 22 has been sequenced by the Human Genome Project. This sequence is available on the internet at: http://www.sanger.ac.uk/HGP/Chr22, Using the NCBI BLAST server, the genomic sequence and genomic arrangement of CLINT was determined. CLINT cDNA aligned with a single section of genomic sequence on chromosome 22q13.2-13.3 with 99%-100% identity. We have submitted the CLINT cDNA sequence to GenBank, Accession No. AF303887. Thus we conclude based on FISH localization of CLINT to a single chromosome and the presence of only one region on this chromosome which contains CLINT sequence that CLINT is encoded by a single gene. Based on genome BLAST results, the coding region of CLINT spans 170kb and is interrupted by 13 introns.

3.4 CLINT is Highly Expressed in Striated Muscle

Northern blot analysis of a poly A^+ RNA blot from various human tissues (Clontech) was probed with the 700bp 3B7 cDNA. Three transcripts of varying sizes (approximately 1.7kb, 2.0kb, and 6.0kb) were discovered which were highly expressed in skeletal muscle and cardiac muscle. In the brain only the large transcript, 6.0kb, appeared to be expressed while the remaining tissues, placenta, lung, liver, kidney, and pancreas, all had lower levels of expression of the three transcripts. This pattern of expression follows the ILK1 expression pattern (not shown) where ILK1 is more highly expressed in the heart and skeletal muscle. ILK1 is also widely expressed in other tissues but barely detectable in the brain. CLINT cDNA was used to probe a Clontech's muscle poly A^+ mRNA Northern blot. Three transcripts were once again identified of sizes 1.7kb, 2.0kb, and 6.0kb. All three were highly expressed in the heart and skeletal muscle while lower levels of expression were seen in the remaining smooth muscle tissues. The lowest transcript, 1.7kb, most likely represents the potential full length cDNA clone, which is 1.6kb in length thus representing CLINT. In light of the genomic analysis of

CLINT, it seems likely these three transcripts arise due to alternative splicing. However an alternate possiblity exists for the presence of the two lower transcripts. These two transcripts differ by approximately 300bp which corresponds to the length of the extended 3'UTR region seen in some CLINT clones. Therefore it is possible that the two lower transcript sizes arise due to alternative polyadenylation resulting in 300bp smaller transcript.

Clontech's multi tissue array poly A^+ mRNA was probed also with [^{32}P] labelled CLINT cDNA. Once again, the highest expression was seen in skeletal muscle and cardiac muscle. Interestingly, CLINT mRNA expression in fetal heart is dramatically lower than that in adult heart suggesting that CLINT is needed in the mature heart muscle.

3.6 CLINT antibodies recognize a ca. 33 kDa cellular protein

Rabbit polyclonal anti-3B7 antibodies were produced by immunizing New Zealand White rabbits with a 3B7-His fusion protein. The rabbit anti-3B7 antiserum, but not the preimmune rabbit serum, strongly reacted with 3B7-His fusion protein in immunoblotting. Cell lysates from HEK 293 cells were analyzed for the presence of CLINT using affinity purified 3B7 antibody. A strong band at 33kDa is seen in HEK 293 lysates which we believe represents CLINT. Upon longer exposure of Western blot to film (> 5 minutes) a faint band at 34kDa and faint bands at higher molecular weights are seen. To determine the specificity of the antibody, affinity purified 3B7 antibodies were incubated with excess recombinant 3B7-His peptide or an unrelated His-tagged protein and used to probe HEK293 cell lysate. The band normally seen at 33kDa is not present in the Western blot with 3B7-His peptide adsorbed antibodies but is seen with the anti-3B7 antibodies adsorbed with the unrelated His-tagged protein Western blot. The apparent molecular weight of CLINT determined from SDS-PAGE is lower than the molecular weight predicted by the Gene Inspector program.

4 MATERIALS AND METHODS

4.1 cDNA Libraries and Screening

The placental λgtII phage library was a gift from Dr Morag Park, McGill University. The skeletal muscle phage library and the Human Heart Matchmaker GAL4 cDNA library (Clontech) were a gift of Paula Demacio and Dr. Peter Ray, Hospital for Sick Children. Both the placental and muscle Rapid Screen cDNA libraries were purchased from Origene. Radioactive nucleotides were from Amersham Corporation. cDNA probes were labelled with [^{32}P] by T7 Quick Prime Kit from Amersham Pharmacia Biotech. Restriction enzymes, T4 ligase, and isopropyl β-thiogalactopyranoside (IPTG) were purchased from MBI Fermentas. Chemical reagents were purchased from Sigma. MBP used in kinase assays was a gift from Dr. Mario Moscarello, Hospital for Sick Children. Sigma's Plasmid Miniprep Kits were used for small scale plasmid purification (1-5mL of bacteria culture) while Promega's midiprep kit was used for large scale plasmid purification (50-100mL of bacterial culture). PCR (Polymerase Chain Reaction) purification was carried out using Qiagen PCR Purification Kit. Isolation of DNA from agarose gels was performed using Qiagen's Gel Extraction Kit. All agarose gels were 1% agarose in 1x TBE (90mM TRIS, 90mM boric acid, 2mM EDTA) and run in 1x TBE. Nucleic acid and protein databases were searched with DNA and protein sequences using the BLAST server of the National Centre for Biotechnology Information (NCBI). Sequence alignments were carried out using web-based programs (www2.ebi.ac.uk/clustalw/ and www.toulouse.inra.fr/multialin.html). Protein motif databases were searched using Prosite and Pfam (www.expasy.ch.tools/).

4.2 Polymerase Chain Reaction Protocols

Primers for PCR were synthesized by GibcoBRL using their oligonucleotide synthesis service (50nM, deprotected oligonucleotides). Cloned Pfu polymerase from Stratagene was used for high-fidelity PCR when the DNA was to be cloned into a vector. Routine PCR was carried out by Qiagen Taq (1 unit of Taq/ 20μL PCR reaction volume) using their Q buffer and following standard protocol in Perkin Elmer cyclers 9600 or 2400 (94°C for 10 minutes(m), 20 cycles on 94°C for 75 seconds(s), 65°C Δ -0.5°C for 1m, 72°C for 2m, 10 cycles on 94°C for 75s, 55°C for 1m, 72°C for 2m, followed by 72°C for 10m).

4.3 DNA Sequencing

DNA was sequenced using ^{33}P and the Thermosequenase kit from Amersham Life Science. The DNA was denatured (2mM EDTA and 2M sodium hydroxide), then precipitated with 10mM sodium acetate and 2.5x vol. of 100% ethanol at -80°C. The DNA pellet was washed with 70% ethanol before being resuspended in water. Each sequencing reaction (3μg DNA, 100ng primer, 8 units thermosequenase, 1x reaction buffer) was divided into 4 tubes containing dGTP master mix and 0.23μCi of either [^{33}P]dATP, [^{33}P]dCTP, [^{33}P]dGTP or [^{33}P]dTTP. The sequencing reaction was cycled (95°C for 30s, 60°C for 30s, 72°C for 1m for 30 cycles) on a Perkin Elmer 9600 or 2400 thermocycler. For sequences with rich GC content dITP was used instead of dGTP master mix and cycled at 95°C for 30s, 55°C for 30s and 60°C for 5m for 30 cycles. Sequencing gels (6% acrylamide and 8.3M urea in 1x TBE) were either run for 6 hours or 3 hours at 1500V. Sequencing gels were dried for 2 hours at 80°C on BioRad drier Model 583 and exposed to Kodak X-OMAT imaging film overnight at room temperature with two intensifiying screens. Some DNA samples were also sent to ACGT Corporation (700 Bay Street, Suite 2230. Toronto, Ontario. M5G 1Z6) for automated sequencing.

4.4 Plasmid Construction

To produce 6xHis-tagged fusion 3B7 peptides, pProEX expression plasmids (Life Technologies) containing 3B7 cDNA were constructed (pProEx-3B7). The 3B7 cDNA was excised with EcoR1 and Xho1 from the yeast two-hybrid isolated plasmid pJG4-5/3B7, which contained 3B7 cDNA. The 3B7 cDNA insert was ligated into the EcoR1/Xho1 site of the pProEX-HTC plasmid so 3B7 cDNA was in frame with the 6xHis tag and then expressed in BL21 DE(3) E. coli strain. The plasmid pProEx/3B7 was sequenced to confirm the sequence and orientation of the 3B7 cDNA insert.

To produce ILK1-GST (glutathione S-transferase) fusion protein, ILK1 was cloned into a pGEX vector. ILK1 cDNA was excised from plac1 clone obtained from a placental cDNA library and inserted in frame into the EcoR1 site of pGEX-4T-1 plasmid (Pharmacia) and transformed into BL21DE(3) E. coli strain. ILK1 was mutated at

E359K by site-directed mutagenesis to create kinase dead ILK1 (ILK1kd). This kinase dead ILK1 was made by Chungyee Leung-Hagesteijn, Hospital for Sick Children. ILK1kd cDNA was inserted in frame into the EcoR1 site of pGEX4T-1 to create ILK1kd-GST fusion vector.

3B7 was inserted into pIND/V5-His-TOPO mammalian vector (Invitrogen) to produce V5-His fusion protein. The PCR products produced using 3B7 cDNA as a template and the 3B7 specific primers (5'ACGATGGCGTCCAGCCACAT3' and 5'CTCCACGTTCTTGTACTTGG3') were directly cloned into the linearized pIND/V5-His-TOPO vector. This vector contains single 3' thymidine (T) overhangs which bind to the single deoxyadenosine (A) added to the 3' ends on the PCR products by *Taq* polymerase. The ligated vector containing the PCR product insert was transformed into DH5α E. coli strain. The new construct was sequenced to confirm sequence and orientation before being used to transfect mammalian cells.

The reverse transcriptase polymerase chain reaction (RT-PCR) products were directly cloned into the linearized TA Cloning vector pCRII (Invitrogen) using the TA cloning Kit (Invitrogen) according the company's protocol. This vector contained single 3' T- overhangs which bound to the single A residues added to the ends of the RT-PCR products by Advantage Taq 2 (Clontech). The ligated products were then transformed into TOP 10 competent E. coli (Invitrogen) and transformants were selected on medium containing ampicillin and X-gal. The constructs were sequenced on both strands.

4.5 Cell Culture

Human 293 embryonal kidney cells (HEK 293) were obtained from Dr. M. Moran, BBDMR, University of Toronto. These cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin/streptomycin, 0.1mM nonessential amino acids, and 2mM glutamine at 37°C as before. HEK 293 cells were detached by gently tapping the flask to dislodge cells, washed in 1x PBS and lysed with NP40 lysis buffer as described above.

4.6 Screening of cDNA Libraries

To isolate a full length cDNA of 3B7 a variety of libraries were screened. Phage muscle and placental libraries were introduced into *E. Coli* Y1088 strain in the presence of 10mM Mg_2SO_4 and 0.2% maltose before plating onto LB+Ampicillin plates. Approximately 500 000pfu (50,000 pfu/plate) were screened by lifting plaques onto Hybond-N^+ nylon membranes (Amersham Life Science). The membranes were hybridized (6x SSC, 5x Denhardt's Solution, 0.5% SDS, 100µg/mL salmon sperm DNA) overnight at 68°C with ^{32}P-labelled 3B7 probe. Membranes were washed (2x SSC, 0.1% SDS) at room temperature followed by washes (0.1x SSC, 0.1% SDS) at 68°C. Positive plaques were isolated and replated to obtain single plaques. These plaques were screened again with the labelled 3B7 probe as above. All plaque lifts were done in duplicate and coincident signals were identified as positive.

The Origene Rapid-Screen cDNA Library Panels are a series of 500 000 arrayed cDNA clones. A tissue specific master plate consisted of 500 000 different cDNA clones in a total of 96 wells at a concentration of 6 ng/µL. Origene muscle and placental Rapid-Screen cDNA Library master plates were screened by PCR using 3′ primers specific for 3B7 (5'GAGGTCCAAGTTAACCAC3′ and 5′CTCCCCCGTCACTCCACGTTCTTG3′) and 5′ primers specific for the pCMV6 plasmid sequence (5'TGGGCGGTAGGCGTGTACGG3' or 5′GCAGAGCTCGTTTAGTGAACC3′) to identify wells with plasmids containing longer cDNA inserts corresponding to 3B7. Positive wells were identified by running PCR products on 1% agarose gel and selecting wells which contained PCR products longer than 1kb. Each well of the master plate corresponded to a subplate which contained only 50 cDNA clones per well in E. coli glycerol stocks. Subplates corresponding to the positive wells identified in the master plate screen were also screened by PCR using the specific primers mentioned above. Positive wells were identified as described before. To isolate desired clones, positive wells were plated onto LB plus Ampicillin plates to obtain single colonies. Ninety-six individual bacterial colonies were picked and dotted onto a gridded master LB plate and into a PCR reaction. The single bacterial colonies were subjected to PCR as described above. The positive bacterial colonies were grown overnight and their plasmids isolated and sequenced.

The human adult heart Matchmaker cDNA library (3.5 x 10^6 independent clones are present in the library) (Clontech) in pACT2 vector was transformed into DH5α and plated on LB+ Ampicillin plates to a concentration of approximately 20 000 cfu/plate. Approximately 200 000cfu were screened by being lifted onto ten 120mmx50mm sterilized nylon membranes and duplicates were made. The membranes were hybridized with ^{32}P-labelled 3B7 cDNA probe in Express Hyb (Clontech) for 1 hour at 68°C. The membranes were washed (2x SSX, 0.05% SDS) at room temperature followed by washes (0.1x SSC, 0.1% SDS) at 50°C before being exposed to film overnight at –80°C. Secondary screens were performed on single colonies obtained from positive colonies identified in the primary screen. Positive clones from the secondary screen were grown in LB+Amp overnight and the plasmid DNA was isolated and sequenced.

Based on BLAST searches of the Genbank expressed sequence tag (EST) database three image clones (IMAGE clones 74203, 167851, and 62240) were ordered and sequenced in hopes of obtaining a full length clone.

4.7 RT-PCR and Cloning of Full Length cDNA Candidate

Total RNA was isolated from Rhabdomyosarcoma cell line by incubating cells with Trizol Reagent (GibcoBRL), adding chloroform and collecting the aqueous layer. Isopropanol was added to precipitate RNA which was pelleted, washed and resuspended in DEPC-H2O.

BLAST searches using 3B7 cDNA identified the presence of a 1347bp sequence in the database, CGI-56 (Accession number AF151814), which was identical to 3B7 in its 3' terminus. Primers were designed based on the 5' end of CGI-56 (5'GAGATGAAGAAGGACGAGTCGT3', 5'GAGGTGAGTGACCTGCAGGAAG3') and on 3B7 (5'CTCCCCCGTCACTCCACGTTCTTG3') and used in RT-PCR on RNA isolated from rhabdomyosarcoma cell line. First strand synthesis of cDNA was performed using Clontech's RT-PCR kit and the 3' specific primer. RT-PCR was performed using the specific primers and Clontech's Advantage 2 Taq. The PCR products were ligated into the TA cloning vector pCRII (Clontech), and sequenced to confirm their identity.

The Clontech human adult heart library was screened with the ^{32}P-labelled RT-PCR product corresponding to a longer cDNA representing 3B7. From this screen we isolated a 1589bp cDNA, 4HiA, that was subsequently determined to correspond to the full length of 3B7 and code for a protein we have called CLINT.

4.8 Northern Blots

A multi tissue poly A^+ mRNA Northern blot (Clontech) was hybridized with a [^{32}P]-dCTP labelled 3B7 probe prepared using the T7 Quick Prime Kit from Amersham. The Northern blot contained equal amounts of poly A^+ mRNA from human heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis. Hybridization was carried out using Clontech's ExpressHyb for 1 hour at 68°C. The blot was washed (2x SSC, 0.1% SDS) at room temperature followed by washes (0.1x SSC, 0.1%SDS) at 50°C. Hybridization of the probe with mRNA bands was visualized by autoradiography at minus 70°C with intensifying screens.

A ^{32}P-labelled CLINT cDNA probe was used to probe a Multi Tissue Array Blot from Clontech, a Muscle Northern Blot containing mRNA from a variety of muscle tissues (cardiac muscle, skeletal muscle, uterus, colon, small intestine, bladder, stomach , and prostate) and a poly A^+ mRNA Mouse Embryo Multi-Tissue Northern Blot (Clontech). The multi-tissue array and the Muscle Multi Tissue Northern were hybridized with the CLINT cDNA probe in ExpressHyb as described above and visualized by autoradiography at -70°C with two intensifying screens.

4.9 Expression of Recombinant His-Tagged and GST-tagged Fusion Proteins

To generate His-tagged fusion peptides, the E. coli BL21(DE3) cells containing pProEX/3B7, in mid-logarithmic cultures, were induced with 1mM isopropyl β-thiogalactopyranoside (IPTG) and harvested several hours later by centrifugation (4500xg). The bacteria (in 1mM PMSF, 50mM Tris, 300mM KCl) were lysed by sonication and centrifuged (9000xg) to remove debris. The 6xHis-tagged fusion protein was affinity purified using Ni^+-NTA beads (Qiagen) according to company's protocol with the exception of an increase in the imidazole and potassium chloride concentration

in the wash buffer to 40mM and 300mM, respectively and to 100mM and 300mM in the elution buffer. Protein concentration was calculated using a 280nm reading according to the formula: 1 OD280nm = 0.5mg/mL of protein.

Prokaryotic expression of ILK1-GST and ILK1kd-GST was induced with 1mM IPTG in mid log cultures and harvested several hours later by centrifugation (4500xg). The bacteria (in 1% triton X100, 25% sucrose, 1mM EDTA, 5mM DTT, 1mg/mL lysozyme in 1x PBS pH 7.4) were lysed by sonication, and centrifuged at 4500 x g to remove debris. The recombinant protein was affinity purified using Glutatione-Sepharose 4B beads (Pharmacia) according to the Pharmacia's protocol. The purified proteins were stored at -80°C.

4.10 3B7 Antibody Production

Rabbit polyclonal anti-3B7 antibodies were produced by immunizing New Zealand White rabbits with a purified 3B7-His fusion protein (purification carried out as detailed in section 3.13) (Research Genetics in Huntsville AL, USA). The rabbit anti-3B7 antiserum, but not the preimmune rabbit serum, strongly reacted with 3B7-His fusion protein in immunoblotting. The rabbit anti-3B7 antiserum recognized a prominent protein band in HEK293 lysate with an apparent molecular mass of approximately 33kDa. Additionally two protein bands with higher molecular masses were also recognized by the rabbit anti-3B7 antiserum after longer exposure to film. In control experiments, no protein band was recognized by the preimmune rabbit serum. The polyclonal anti-3B7 antibody was affinity purified by incubating the antiserum with 3B7-His peptide attached to Ni^{+}-NTA beads (in 10mM Tris pH8.0 solution) overnight. The beads were first washed with 10mM Tris pH 8.0, then with 500mM NaCl and 10mM Tris pH 8.0. The antibodies were eluted with 100mM glycine pH 3.0 followed by 100mM triethylamine pH 11.5 and neutralized immediately with 1M Tris pH 8.0 to a concentration of 10mM Tris. The eluted antibodies were added to 1x TBS and 1% BSA for use in Western blotting.

The specificity of the anti-3B7 antibody was determined by preabsorbing the purified antibody with 3B7-His tagged fusion protein bound to Ni-resin and to an

unrelated His-tagged protein bound to resin as a control overnight at 4°C. Complexes of anti-3B7 IgG and 3B7-His peptide bound to the Ni^{+}-NTA resin were pelleted and the absorbed serum was used for Western blotting and compared to non-absorbed serum on HEK 293 lysates.

5. REPORTABLE OUTCOMES

5.1 Meeting Abstracts

1. Carnio L, Qiong Y, Leung-Hagesteijn, Hannigan GE. Cloning of a novel actinin-like gene by direct interaction with the integrin-linked kinase, ILK. 38th Annual Meeting of the American Society for Cell Biology, Dec. 12-16, 1998, San Francisco, CA. Poster.
2. Hannigan GE, Naruszewicz I, Leung-Hagesteijn C. Cloning of a novel interactor of the integrin-linked kinase, ILK. Gordon Conference on "Fibronectin, Integrins and other molecules" Jan. 31- Feb. 5, 1999, Ventura CA. Poster.
3. Miller M, Hannigan GE. The role of Integrin-Linked Kinase (ILK-1) Signalling in Myogenesis. Thirty-ninth Annual Meeting of the American Society for Cell Biology, Washington, D.C., Dec. 11-15, 1999. Poster Abstracts, p. 171.
4. Carnio L, Qiong Y, Hannigan GE. CLINT, Identification of CLINT, a novel Calponin homology domain protein that interacts with Integrin-linked kinase, ILK-1. Thirty-ninth Annual Meeting of the American Society for Cell Biology, Washington D.C., Dec. 11-15, 1999. Poster.
5. Leung-Hagesteijn C, Naruszewicz I, Mahendra A, Hannigan G. A Novel Protein Phosphatase 2C Regulating ILK1 Signal Transduction. U.S. Department of Defense Breast Cancer Research Program Meeting, Atlanta, GA, June 8-11, 2000 (poster).

6. Leung-Hagesteijn C, Mahendra A, Naruszewicz I, Hannigan G. Selective Regulation of GSKß Activity by a Novel Protein Phosphatase Associating with Integrin-Linked Kinase, ILK1. Gordon Conference on 'Signalling by Cell Adhesion Receptors', Salve Regina University, RI July 23-28, 2000 (poster).

5.2 Manuscripts

1. Mathew G. Miller, Chungyee Leung-Hagesteijn, Toolsie Ramlal, and Gregory E. Hannigan 'Integrin-linked Kinase-1, ILK1, is required for in vitro differentiation of rat L6 myoblasts' In revision for J. Biol. Chem.

2. Chungyee Leung-Hagesteijn, Ahalya Mahendra[1], Izabela Naruszewicz, Gregory E. Hannigan[1,2]
'Modulation of integrin signal transduction by ILKAP, a protein phosphatase 2C associating with the integrin linked kinase, ILK1'. EMBO J.

5.3 Patents applied for

1. "ILK Interacting Protein CLINT" U.S. Provisional Application
March 25, 1998 (Hannigan).
2. Novel Protein Phosphatase, ILKAP, and Methods of Use Therof.
U.S. Provisional Application, November 30, 1999.

5.4 GenBank submissions

1. ILKAP: Accession AY024365
2. CLINT: Accession AF303887

6. CONCLUSIONS

We have identified two protein interacting partners of integrin linked kinase, ILK1. ILKAP, a protein phosphatase 2C is an important regulator of the Wnt signal transduction pathway, known to be important in breast tumour development and progression. Clint is a putative actin-binding protein that provides a news insight into the

functional linkage between integrins and regulation of actin cytoskeletal changes. These linkages may also affect ILK regulation of E-cadherin function. These proteins represent new targets for anti-neoplastic therapeutics aimed at modulation of ILK signalling.

7. NOTE ON YEAR 2 AND 3 OBJECTIVE

Regarding work originally proposed to develop transgenic mice expressing ILK in the mammary epithelium, I had reason to revise these as follows. Shortly after obtaining this award, I became aware of the fact that Dr. W. J. Muller (McMaster University, Canada) had already started to develop MMTV-ILK mice, exactly as I had proposed. As I was starting up a new lab and Dr. Muller is an established investigator, much experienced in developing MMTV mouse models of breast cancer, I decided not to duplicate Dr. Muller's efforts and to concentrate my efforts on ILK interacting proteins. We are now developing conditional knock-outs in mice of the ILKAP phosphatase, so that questions of its role in mammary (and other) cancer can be evaluated.

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Modulation of integrin signal transduction by ILKAP, a protein phosphatase 2C associating with the integrin-linked kinase, ILK1

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ILKAP, a protein serine/threonine (S/T) phosphatase of the PP2C family, was isolated in a yeast two-hybrid screen baited with integrin-linked kinase, ILK1. Association of ILK1 and ILKAP was independent of the catalytic activity of either partner, as assayed in co-precipitation and two-hybrid experiments. Conditional expression of ILKAP in HEK 293 cells resulted in selective inhibition of ECM- and growth factor-stimulated ILK1 activity, but did not inhibit Raf-1 kinase activity. A catalytic mutant of ILKAP, H154D, did not inhibit ILK1 kinase activity. Two cellular targets of ILK1, glycogen synthase kinase 3 β (GSK3β) and protein kinase B (PKB)/AKT, were differentially affected by ILKAP-mediated inhibition of ILK1. Catalytically active, but not mutant ILKAP, strongly inhibited insulin-like growth factor-1-stimulated GSK3β phosphorylation on Ser9, but did not affect phosphorylation of PKB on Ser473, suggesting that ILKAP selectively affects ILK-mediated GSK3β signalling. Consistent with this, active, but not H154D mutant or the related PP2Cα, selectively inhibited transactivation of a Tcf/Lef reporter gene, TOPFlash, in 293 cells. We propose that ILKAP regulates ILK1 activity, targeting ILK1 signalling of Wnt pathway components via modulation of GSK3β phosphorylation.

Keywords: β-catenin/GSK3β/integrin-linked kinase/protein phosphatase 2C/signal transduction

Introduction

Integrin-mediated cell adhesion to extracellular matrices (ECM) stimulates a number of protein kinase activities, such as phosphoinositide-3-OH kinase (PI3K), src family and FAK tyrosine kinases, as well as protein serine/threonine (S/T) kinases such as ILK1 and protein kinase B (PKB) (Dedhar and Hannigan, 1996; Hanks and Polte, 1997; Hannigan and Dedhar, 1997; Banfic *et al.*, 1998; Dedhar, 1999). Integrin-linked kinase, ILK1, functions to mediate integrin signal transduction, and was originally identified in a yeast two-hybrid screen for protein partners of the β1 integrin subunit cytoplasmic domain (Hannigan *et al.*, 1996). ECM and growth factors each stimulate rapid, transient activation of ILK1 activity and signalling. Failure to down-regulate ILK1-mediated signalling can lead to oncogenic transformation, since constitutive over-expression of $p59^{ILK1}$ induces anchorage-independent growth of epithelial cells, reflecting induction of cyclin D1/cdk4 expression and activation (Hannigan *et al.*, 1996; Radeva *et al.*, 1997). More recently, it has been demonstrated that ILK1 stimulation by ECM or growth factors is dependent on the activity of PI3K, a widely acting mediator of cell growth and survival signals. Moreover, ILK1 is activated *in vitro* by phosphatidylinositol (3,4,5) trisphosphate (PIP_3), suggesting physiological activation of ILK1 by lipid products of PI3K activity (Delcommenne *et al.*, 1998; Morimoto *et al.*, 2000).

A number of studies have shown that integrin-mediated cell adhesion stimulates PI3K-dependent activation of PKB (Khwaja *et al.*, 1997; Banfic *et al.*, 1998). A recent report on the inverse correlation between PIP_3 lipid phosphatase (PTEN) and PKB expression in a series of primary acute leukaemias and non-Hodgkin's lymphomas provides additional support for the role of PI3K signalling in regulating tumour progression and survival via PKB (Dahia *et al.*, 1999). Phospholipid-dependent kinase-2 (PDK2) activity effects Ser473 phosphorylation and it has been shown that ILK1 stimulates Ser473 phosphorylation (Delcommenne *et al.*, 1998; Lynch *et al.*, 1999). Conversely, phosphorylation on Thr308 of PKB is effected by a distinct phospholipid-sensitive PDK1, both these phosphorylations being required for full activation of PKB. Although ILK1 is a candidate PDK2 regulating PKB-mediated cell survival, ILK1 has also been shown to target glycogen synthase kinase 3 β (GSK3β) (Delcommenne *et al.*, 1998; D'Amico *et al.*, 2000; Persad *et al.*, 2000). GSK3β is an important mediator of developmental signalling of the Wnt/wingless pathway, affecting the transcriptional activity of Tcf/Lef factors through phosphorylation of the transcriptional co-factor β-catenin (Barker *et al.*, 2000). GSK3β phosphorylation of β-catenin targets the latter for degradation, and overexpression of ILK1 in epithelial cells induces an inhibitory phosphorylation of GSK3β at Ser9 (Delcommenne *et al.*, 1998; D'Amico *et al.*, 2000; Tan *et al.*, 2001), resulting in stabilization and nuclear translocation of β-catenin, with concomitant activation of Tcf/Lef factors (Novak *et al.*, 1998; Dedhar *et al.*, 1999). Thus, ILK1 is emerging as an important intracellular regulator of signalling via components of the Wnt/wingless pathway, specifically through modulation of GSK3β activity.

In this paper we describe the isolation of a novel member of the protein phosphatase 2C (PP2C) family in a yeast two-hybrid screen to identify proteins that interact with $p59^{ILK1}$. We have named this PP2C family member ILKAP, for ILK1-associated phosphatase. ILK1 and ILKAP were co-precipitated from lysates of HEK 293

cells, independently of ILK1 or ILKAP catalytic activities. Induced expression of recombinant, catalytically active ILKAP in HEK 293 cells resulted in the inhibition of protein kinase activity in ILK1 immune complexes. Conversely, ILKAP did not affect Raf-1 immune complex kinase activity, and a catalytically inactive ILKAP mutant did not inhibit ILK1 activity. ILKAP expression strongly inhibited integrin-stimulated phosphorylation of GSK3β at Ser9; however, we did not observe an ILKAP-mediated block to integrin-induced phosphorylation of PKB. Finally, we show that ILKAP selectively inhibited activation of Tcf/Lef factors, consistent with the observed inhibition of GSK3β Ser9 phosphorylation. These results suggest that ILKAP complexes with ILK1 to selectively inhibit the GSK3β arm of ILK1 signalling. Taken together, our data describe a new cytoplasmic protein S/T phosphatase, ILKAP, that down-regulates integrin- and growth factor-stimulated ILK1 activity, and lead us to propose that ILKAP is a physiological regulator of ILK1 signalling.

Results

Identification and characterization of ILKAP

A yeast two-hybrid screen was performed, using a bait plasmid encoding a C-terminally truncated ILK1 protein comprising amino acid residues 1–276 (Hannigan *et al.*, 1996). This construct contained the N-terminal ankyrin-like repeat domain of ILK1, the pleckstrin homology-like domain and the first few residues of the catalytic domain. One positive clone identified in a screen of HeLa cell cDNAs yielded a 700 bp cDNA (designated 5BT) possessing significant sequence similarity to S/T phosphatases of the PP2C family. Oligonucleotide primers derived from this cDNA sequence were synthesized and used to amplify clones from human placental and skeletal muscle cDNA libraries (OriGene Rapid Screen; see Materials and methods). A cDNA of 1.7 kb was isolated (Figure 1A), exhibiting a high degree of similarity to PP2C family phosphatases (Figure 1B). Northern blot analyses of human multi-tissue and muscle RNA blots indicated that this probably represented a full-length transcript (Figure 2). The 1.7 kb cDNA contains on open reading frame encoding a protein of 392 amino acid residues. The major features of this coding region are two PP2C boxes: one comprising residues 115–206 (PP2C box 1) and the second encompassing residues 215–392 (PP2C box 2). This architecture is typical of PP2C family members (Wera and Hemmings, 1995; Barford *et al.*, 1998), and within these PP2C boxes highly conserved catalytic residues are present in the ILK1-trapped sequence. In particular, a DGH triplet within PP2C box 1 is absolutely conserved in all PP2Cs and represents the catalytic active site, as shown by mutagenesis and X-ray crystallography studies (Das *et al.*, 1996; Sheen, 1998). Accordingly, we called the trapped PP2C, ILKAP. The deduced ILKAP amino acid sequence displays a high degree of similarity (95% identity) to rat PP2C, described by Tong *et al.* (1998), suggesting that ILKAP is the human homologue of rat PP2C.

Northern blot analyses of human multi-tissue blots indicated that ILKAP is widely expressed, with the highest transcript levels expressed in striated muscle (data not shown). Since ILK is also important for muscle development in *Caenorhabditis elegans* (Dedhar *et al.*, 1999), we examined ILKAP expression in human muscle tissue blots. ILKAP was preferentially expressed in striated muscle, with much lower levels evident in various smooth muscle tissues (Figure 2A). Dot-blot analyses of multi-tissue arrays containing 56 tissue RNAs indicated that ILKAP is ubiquitously expressed, and verified highest expression levels in cardiac and skeletal muscle, similar to ILK1 (not shown). In order to characterize an ILKAP-encoded protein, we generated a His_6-tagged recombinant fusion protein by subcloning the 700 bp 5BT, C-terminal cDNA into pProExHT for expression and purification in *Escherichia coli*. Soluble recombinant protein was purified on Ni-NTA columns, and used to immunize rabbits. Affinity-purified ILKAP antibodies recognize a protein band migrating with an apparent mol. wt of 47 kDa, as estimated by SDS–PAGE, similar to the molecular weight of characterized PP2C family members (Figure 2B). These affinity-purified ILKAP antibodies recognized $p47^{ILKAP}$ in ILK1 immunoprecipitates of 293 cell lysates, indicating that $p59^{ILK1}$ and $p47^{ILKAP}$ associate under physiological conditions (Figure 2B).

In order to investigate whether ILKAP is a functional PP2C, we expressed recombinant ILKAP proteins in eukaryotic cells, and characterized the catalytic activity of these proteins using *in vitro* phosphatase assays (Figure 3). For this purpose we cloned full-length ILKAP cDNAs, representing wild-type and mutant (see below) proteins, into the pIND/V5TOPO-His expression plasmid. When co-transfected with pVgRXR, encoding a modified ecdysone receptor, pIND/V5-ILKAP conferred ecdysone (muristerone A)-inducible expression of V5-tagged ILKAP in transiently transfected 293 cells (Figure 3). Control phosphatase assays indicated that 85–90% of the total protein S/T phosphatase activity in HEK 293 cells was sensitive to okadaic acid (OA) and EGTA, inhibitors of PP1, PP2A and PP2B phosphatases (data not shown). Muristerone-inducible PP2C (ILKAP) activity was thus assayed *in vitro* in the presence of OA and EGTA, using PKA-phosphorylated, ^{32}P-labelled myelin basic protein (MBP) as substrate. Vector (pVgRXR) control HEK 293 transfectants showed no muristerone-inducible PP2C activity; however, the ILKAP transfectants showed ~2.5-fold induction of PP2C activity (Figure 3A). Furthermore, ILKAP activity was inhibited by 50% in the presence of Mg^{2+} ions, as has been reported for rat PP2C (Tong *et al.*, 1998). These assays produced only minimal background activity in the absence of Mn^{2+} ions (not shown), further indicating that the ILKAP cDNA encodes Mn^{2+}-dependent PP2C activity.

To characterize ILKAP catalytic activity further, mutant H154L and H154D proteins were generated by site-directed mutagenesis of the putative active site, ^{152}DGH^{154} (see Materials and methods). To assay for catalytic inactivation of these ILKAP mutants, HEK 293 cells were transiently transfected with pVgRXR and pIND/V5-ILKAP, or pIND/V5-ILKAP mutant cDNAs. Three independently constructed mutant ILKAP plasmids, two encoding H154L and one encoding H154D, were transfected in parallel and assayed for phosphatase activity, as above. Wild-type ILKAP transfectant cells demonstrated muristerone-inducible PP2C activity; however, despite

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1   MDLFGDLPEPERSPRPAAGKEAQKGHLLFDDLPPASSTDSGSGGPLLFDD
51  LPPASSGDSGSLATSISQMVKTEGKGAKRKTSEEEKNGSEELVEKKVCKA
101 SSVIFGLKGYVAERKGEREEMQDAHVILNDITEECRPPSSLITRVSYFAV
151 FDGHGGIRASKFAAQNLHQNLIRKFPKGDVISVEKTVKRCLLDTFKHTDE
201 EFLKQASSQKPAWKDGSTATCVLAVDNILYIANLGDSRAILCRYNEESQK
251 HAALSLSKEHNPTQYEERMRIQKAGGNVRDGRVLGVLEVSRSIGDGQYKR
301 CGVTSVPDIRRCQLTPNDRFILLACDGLFKVFTPEEAVNFILSCLEDEKI
351 QTREGKSAADARYEAACNRLANKAVQRGSADNVTVMVVRIGH
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B

```
                    *         20         *         40         *         6
ILKAP       : KGER------------------------EEMQDAHVILNDITEECRPPSSLITRVSYF :  34
PP2Calpha   : MGAFLDKPKMEKHNAQGQGNG---LRYGLSSMQGURVEMEDAHTAVIGLPSGLESWSFF :  56
PP2Calpha2  : MGAFLDKPKMEKHNAQGQGNG---LRYGLSSMQGURVEMEDAHTAVIGLPSGLESWSFF :  56
PP2Cgamma   : MGAYLSQPNTVKCSGDGVGAPRLPLPYGFSAMQGURVSMEDAHNCIPELDS---ELAMF :  56

            0         *         80         *        100         *         1
ILKAP       : AVFDGHGGIRASKFAAQNLHQNL---------------------------------- :  57
PP2Calpha   : AVYDGHAGSQVAKYCCEHLLDEIITNNQDFKGS---------------------------- :  88
PP2Calpha2  : AVYDGHAGSQVAKYCCEHLLDEIITNNQDFKGS---------------------------- :  88
PP2Cgamma   : SVYDGHGGEEVALYCAKYLPDIIKDQKAYKEGKLQKALEDAFLAIDAKLTTEEVIKELA : 115

            20         *        140         *        160         *
ILKAP       : -IRKFPKGDVISVEKTVKRCLLDTFKH---------TDEEFLKQASSQKPAW-------- :  99
PP2Calpha   : --AGAPS--------------VENVKNGI--RTGFLEIDEHMRVMSEKKHGA-------- : 122
PP2Calpha2  : --AGAPS--------------VENVKNGI--RTGFLEIDEHMRVMSEKKHGA-------- : 122
PP2Cgamma   : QIAGRPTEDEDEKEKVADEDDVDNEEAALLHEEATNTIEELLTRYGQNCHKGPPHSKSG : 174

            180         *        200         *        220         *
ILKAP       : -----KDGSTATCVL-----------------AVDNILYIAN----------------- : 119
PP2Calpha   : ----DRSGSTAVGVLISPQ-----------------HQYFIN----------------- : 143
PP2Calpha2  : ----DRSGSTAVGVLISPQ-----------------HQYFIN----------------- : 143
PP2Cgamma   : GGTGEEPGSQGLNGEAGPEDSTRETPSQENGPTAKAVTGFSSNSERGTEAGQVGEPGIP : 233

             240         *        260         *        280         *
ILKAP       : -------LGDSRAILCRY-------------------------------------NE : 132
PP2Calpha   : -------CGDSRGLLCRNRKVHFFTQDHKPSNPLEKERIQNAGGS------------- : 181
PP2Calpha2  : -------CGDSRGLLCRNRKVHFFTQDHKPSNPLEKERIQNAGGS------------- : 181
PP2Cgamma   : TGEAGPSCSSASDKLPRVAKSRFFEDSEDESDEAEEEEEDSEECSEEEDGYSSEEAENE : 292

              300         *        320         *        340         *
ILKAP       : E------------------------------------------------------- : 133
PP2Calpha   : -------------------VMIQRVNGSLAVSRALGDFDYKC-VHGK-------GPTEQ : 213
PP2Calpha2  : -------------------VMIQRVNGSLAVSRALGDFDYKC-VHGK-------GPTEQ : 213
PP2Cgamma   : EDEDDTEEAEEDDEEEEEEEMIVPGMEGKEEPGSDSGTTAVWALIRGKQLIVANAGDSRC : 351

               360         *        380         *        400         *
ILKAP       : --SQKHAALSLSKEHNPTQYEERMRIQKAGGNVR-DGRVLGVLEVSRSIGDGQYKRCGV : 189
PP2Calpha   : LVSPEPEVHDIERSEEDDQFIILA----CDGIWDVMG--------MEELCDFVRSRLEV : 260
PP2Calpha2  : LVSPEPEVHDIERSEEDDQFIILA----CDGIWDVMG--------MEELCDFVRSRLEV : 260
PP2Cgamma   : VVSEAGKALDMSYDHKPEDEVELARIKNAGGKVIMDGRVNGGLNLSRAIGDHFYKRNKN : 410

                 420         *        440         *        460         *
ILKAP       : I--------SVPDIRRCQLTPNDRFILLACDGLFKVFTPEEAVNFI---------- : 227
PP2Calpha   : LDDLEKVCNEVVDTCLYKGSRDNMSVILICFPNAPKVSPEAV-----KKEAELDKYLEC : 314
PP2Calpha2  : LDDLEKVCNEVVDTCLYKGSRDNMSVILICFPNAPKVSPEAV-----KKEAELDKYLEC : 314
PP2Cgamma   : LPPEEQNISALPDIKVLILTDDHEFNVIACDGIWNVMSSQEVVDFIQSKISQRDEMGEL : 469

                  480         *        500         *        520         *
ILKAP       : --LSQLEDEKIQ-------TRECKSAADARYEAACNRLANKAVQR--GSADNVTVMVVR : 275
PP2Calpha   : R-----VEEIIKK--------QGEGVPDLVEVMRTLASENIPSLPPGGELASKRNVIEA : 360
PP2Calpha2  : R-----VE-------------------------------------GGSFNKK------ : 324
PP2Cgamma   : RLLSSIVEELLDQCLAPDTSGDGTGCDNMTCIIICFKPRNTAELQP----ESGKRKLEE : 524

                  540         *
ILKAP       : IGH------------------- : 278
PP2Calpha   : WYNRLNPYKNDDTDSTSTDDMU : 382
PP2Calpha2  : ---------------------- :   -
PP2Cgamma   : VLSTEGAEENGNSDKKKKAKRD : 546
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robust induction of recombinant mutant proteins, PP2C activity in the mutant transfectants was equal to the background activity assayed in the pVgRXR/293 control transfectants (Figure 3B). Taken together, these structural and functional data strongly support the identity of ILKAP as a PP2C.

Specific association of ILK1 and ILKAP is independent of ILKAP catalytic activity

Having demonstrated that the ILKAP cDNA encoded a functional PP2C, it was important to confirm and further characterize the association of ILK1 and ILKAP proteins in mammalian cells. In the transient pIND/V5-ILKAP transfectants, maximal induction of V5-ILKAP was seen at 1 μM muristerone A (Figure 4A), and expression was clearly seen at 6 h post-induction. After 36 h, higher levels of V5-ILKAP accumulation were evident. Therefore, we examined the association of V5-ILKAP with endogenous p59^{ILK1} at these times (Figure 4B). Affinity-purified ILK1 antibodies were used to immunoprecipitate cytoplasmic lysates of uninduced, 6 h and 36 h muristerone-induced transfectants. At 6 h induction we could not detect co-precipitating V5-ILKAP; however, after 36 h, significant levels were detected in the immune complexes

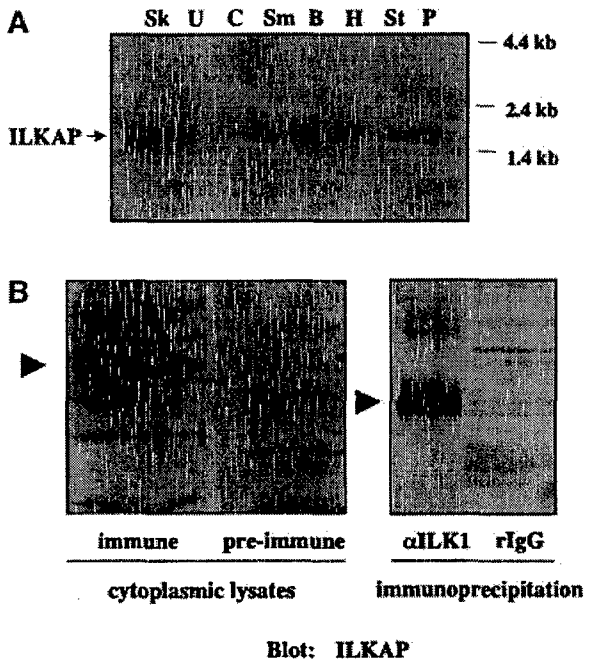

Fig. 2. ILKAP transcript is preferentially expressed in skeletal muscle. (A) A partial cDNA representing amino acid residues 269–392 of ILKAP was ^{32}P labelled, and used to probe a northern blot of poly(A)$^+$ RNAs from human striated and smooth muscle tissues. Sk, skeletal muscle; U, uterus; C, colon; Sm, small intestine; B, bladder; H, heart; St, stomach; P, prostate. (B) Left panel: a polyclonal antibody was raised to an ILKAP fusion protein (residues 269–392) and affinity purified over an immobilized ILKAP column. Pre-immune serum from the same rabbit was subjected to the same purification protocol, as a negative control. Cytoplasmic lysates of HEK 293 cells were analysed by western blotting using the ILKAP immune and pre-immune sera as indicated. Right panel: cytoplasmic 293 cell lysates were immunoprecipitated with rabbit IgG, or affinity-purified rabbit polyclonal ILK1 antibody, covalently coupled to CNBr-activated Sepharose beads. Unfractionated cytoplasmic lysates and ILK1 immunoprecipitates each indicated a single ILKAP band, migrating with an apparent mol. wt of 47 kDa. Immune complexes were analysed by western blotting, using affinity-purified ILKAP antibody. Arrowheads indicate p47^{ILKAP} migration on 12% SDS–PAGE.

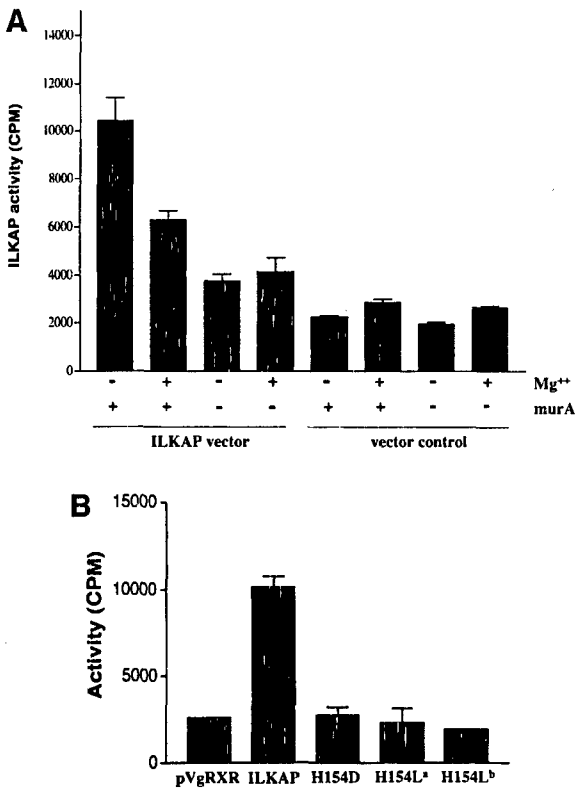

Fig. 3. PP2C activity of recombinant ILKAP expressed in mammalian cells. (A) HEK 293 cells stably expressing the synthetic ecdysone receptor (pVgRXR) were transiently transfected with pIND/V5-ILKAP, in order to characterize muristerone A-induced phosphatase activity. Vector control cells were the stable pVgRXR 293 transfectants. Muristerone A treatment of the pIND/V5-ILKAP cells induced OA- and EGTA-resistant PP2C ~3-fold relative to uninduced pIND/V5-ILKAP transfected cells; however, it did not raise PP2C activity of vector control cells above background. Muristerone-induced S/T phosphatase activity was partially sensitive to inhibition by Mg^{2+}, as reported for rat PP2C (Tong *et al.*, 1998). (B) pVgRXR-expressing 293 cells were transiently transfected with pIND/V5-ILKAP plasmids encoding wild-type ILKAP, or the indicated ILKAP catalytic mutants. Muristerone-induced PP2C activity from the wild-type ILKAP transfectants was ~5-fold above background levels seen with the mutant ILKAP transfectants. All assays were performed in triplicate, with error bars indicating 1 SEM. Control experiments confirmed that this phosphatase activity was dependent on Mn^{2+}, and not inhibited by OA or EGTA, as expected of PP2C enzymes. Thus, all assays in (A) and (B) were carried out in the presence of Mn^{2+}, OA and EGTA.

Fig. 1. ILKAP structure and alignment with PP2C. (A) Deduced protein sequence of ILKAP cDNA. Sequence was translated from the H4C11-30 cDNA sequence using Gene Inspector v. 1.5 software (Textco, Inc., New Hampshire). PP2C boxes 1 and 2 are underlined, and bold letters indicate the DGH active site triplet. (B) Amino acid residues 115–392 of ILKAP were aligned using the Clustal V algorithm (Gene Inspector 1.5) and the alignment edited to highlight identities (black) and conserved (shaded) amino acid residues, using Genedoc v. 2.6.01 (www.psc.edu/biomed/genedoc/).

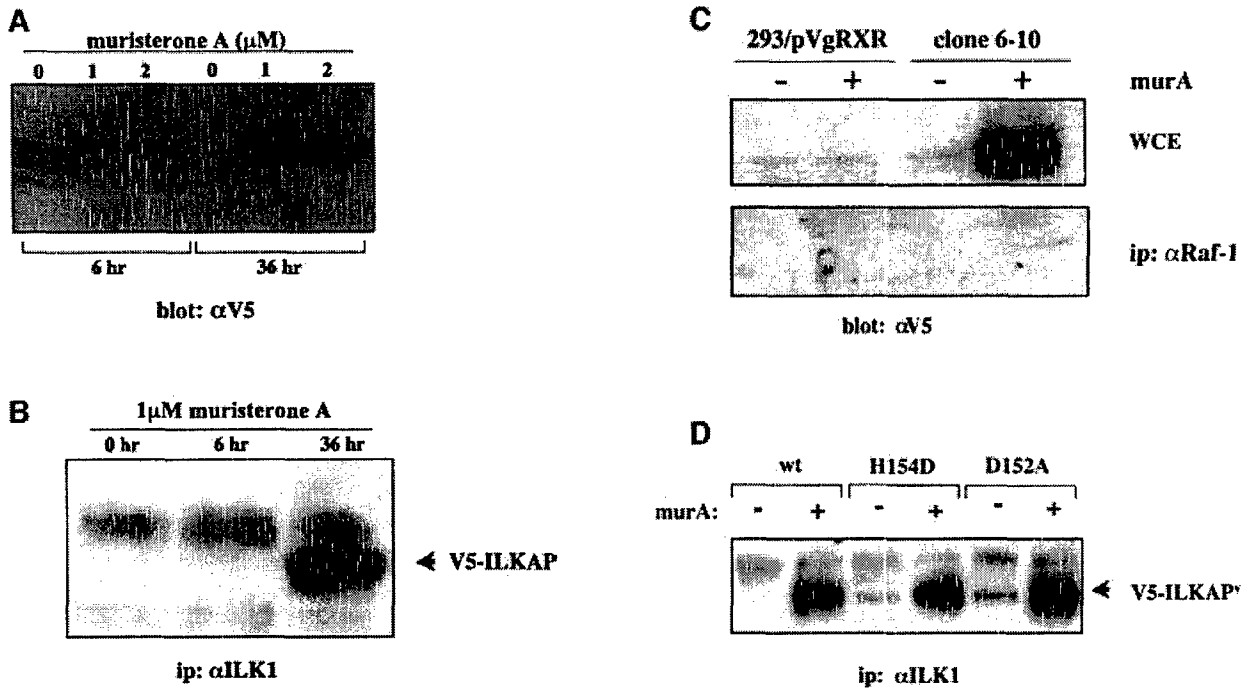

Fig. 4. ILKAP and ILK1 associate in mammalian cells. (**A**) HEK 293 were transiently co-transfected with pIND/V5-ILKAP and pVgRXR plasmids. After 24 h of culture, transfected cells were then induced with 1 or 2 μM muristerone A, or left untreated, for the indicated periods. Cytoplasmic lysates were analysed by western blotting for expression of the V5 epitope, migrating at the appropriate size of ~50 kDa. (**B**) 293 cells were transiently co-transfected, as above. After 24 h, transfectants were induced with muristerone A for the indicated times. Cytoplasmic lysates of these cells were immunoprecipitated with affinity-purified ILK1 antibodies, and the resulting immune complexes analysed by SDS–PAGE and western blotting for the presence of the V5-tagged ILKAP protein. (**C**) 293/pVgRXR and wt/6-10 clones were cultured under normal conditions, prior to induction with 1 μM muristerone A for 36 h. Cytoplasmic lysates (WCE; top panel) were immunoprecipitated (bottom panel) with anti-Raf-1 antibody (C-20; Santa Cruz). Immune complexes were run on 10% SDS–PAGE and transferred to PVDF membranes. Membranes were blotted with anti-V5 monoclonal antibody, and visualization was by ECL. (**D**) 293/pVgRXR cells were transiently transfected as in (A), with the indicated ILKAP mutants. Transfectant cultures were lysed and subjected to immunoprecipitation with affinity-purified ILK1 antibodies. Complexes were analysed for co-precipitating V5-tagged ILKAP and mutants, by SDS–PAGE and western blotting with anti-V5 monoclonal antibody. ILKAP^V indicates variant ILKAP recombinant proteins.

(Figure 4B). V5-tagged ILKAP was not detected in Raf-1 immunoprecipitates of these same lysates (Figure 4C). These results indicate that the observed association of ILKAP and ILK in intact cells is specific, and is not due to a post-lysis artefact. Equally, as we also detect p47^{ILKAP} in ILK1 immune complexes from lysates of untransfected, parental 293 cells (Figure 2B), it is evident that ILK1 association of V5-tagged ILKAP is not a consequence of overexpression and occurs under physiological conditions.

In order to gain additional insight into the regulation of ILK1 by ILKAP, it was important to determine the requirement for ILKAP catalytic activity in the association of p47^{ILKAP} with p59^{ILK1}. Wild-type, H154D and D152A ILKAP plasmids were transiently transfected into pVgRXR-293 cells, which were subsequently induced with muristerone A, and cell lysates subjected to co-immunoprecipitation analyses using affinity-purified ILK1 antibodies. The ILKAP point mutations affect PP2C box 1 and do not directly involve PP2C box 2, which encompasses the ILK1-binding region. The H154D and D152A mutant proteins co-precipitated with p59^{ILK1} as efficiently as the wild-type protein (Figure 4D), demonstrating that phosphatase activity is not required for the physical interaction of p47^{ILKAP} and p59^{ILK1}. These data suggest that integrin-induced signals mediate inactivation of p47^{ILKAP} complexed with p59^{ILK1} to regulate transient ILK1 activation in response to ECM.

ILKAP selectively inhibits ILK1 activity and signal transduction

We next examined the ability of ILKAP to inhibit ILK1 kinase activity and signal transduction. Stable HEK 293 cell lines expressing muristerone A-inducible, V5 epitope-tagged ILKAP were derived for this purpose. Two such independently isolated clones, wt/6-4 and wt/6-10, were chosen for detailed study. In the absence of treatment with muristerone, we could not detect any V5-tagged ILKAP in either wt/6-4 or wt/6-10 clones, indicating that expression was tightly regulated. Cells were untreated or induced with muristerone A for 24 h. These were then re-plated on fibronectin (FN), control bovine serum albumin (BSA), or treated with 100 nM insulin for 20 min to activate endogenous ILK1. Cells were then lysed and subjected to ILK1 immune complex kinase assays. We observed muristerone A-dependent inhibition of ILK1 kinase activity in both wt/6-4 and wt/6-10 lines (Figure 5A). Basal ILK1 activity in these cells is relatively high due to the presence of serum in the cultures, making ECM and insulin stimulation of ILK1 apparently less robust than in serum-starved cultures. ILKAP induction significantly

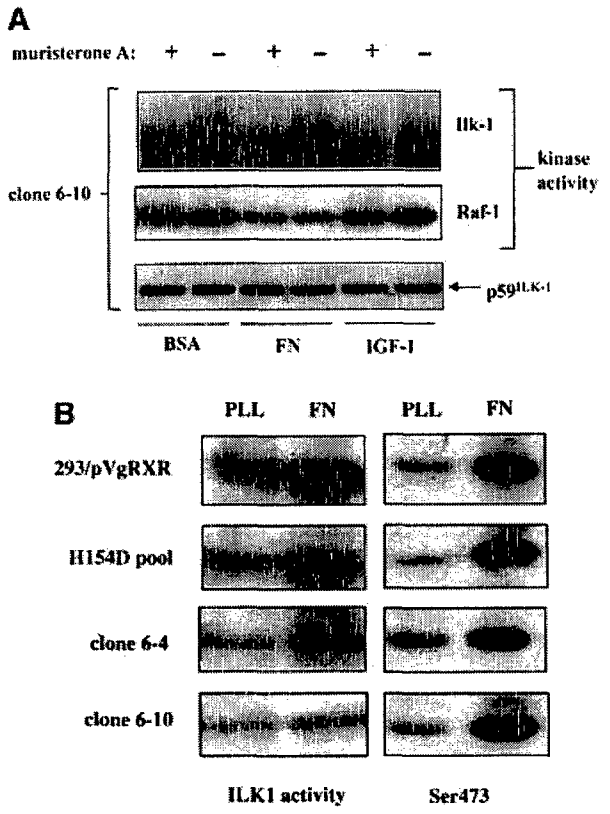

Fig. 5. ILKAP selectively inhibits ILK1 kinase activity.
(**A**) Cytoplasmic lysates from ILKAP clone wt/6-10 were analysed in ILK1 (top panel) or Raf-1 (middle panel) immune complex kinase assays using MBP as exogenous substrate. Cells were pre-treated for 36 h with or without muristerone A, detached, and replated for 20 min on BSA or FN in 10% serum, or treated for 10 min with 100 ng/ml IGF-1, to induce high levels of ILK1 activity. Lysates were controlled for equal levels of $p59^{ILK1}$ protein (bottom panel). (**B**) The stable ILKAP clones wt/6-4 and wt/6-10, and a non-clonal H154D pooled transfectant cell line, were treated with muristerone A for 36 h to induce recombinant protein expression. Subsequently, these cells were plated on FN or poly-L-lysine (PLL) for 10 min, after which cell lysates were assayed for ILK1 activity by immune complex kinase assays (MBP substrate), and also for PKB Ser473 phoshorylation by western blotting with a pSer473-specific antibody. Induction of ILKAP, but not the H154D mutant, inhibited ILK1 immune complex kinase activity. Ser473 phosphorylation was not inhibited in parallel with ILK1 inhibition. Plating on PLL did not induce ILK1 activity or Ser473 phosphorylation.

inhibited the high levels of ILK1 activity observed under these conditions. We wished to confirm that this inhibition was not generalized to other, S/T protein kinases. Raf-1 is a cytoplasmic S/T kinase whose activity is modulated by extracellular signals. Therefore, as a specificity control, we assayed Raf-1 kinase activity in the same lysates used for ILK1 activity. Raf-1 activity was decreased by plating on FN, as expected under conditions where PKB is activated (Zimmermann and Moelling, 1999); however, there was no muristerone A effect on Raf-1 kinase activity, demonstrating that induction of ILKAP selectively inhibited ILK1 immune complex kinase activity.

We next examined the requirement for ILKAP catalytic activity in the observed inhibition of ILK1 activity. A stable, non-clonal cell line was derived from HEK 293 by transfection of the H154D catalytic mutant. Induction of H154D expression did not inhibit ILK1 kinase activity, as seen with ILKAP-expressing wt/6-4 and wt/6-10 cells (Figure 5B). A number of groups have shown that ILK1 activates the PKB survival pathway, by either directly or indirectly effecting phosphorylation of PKB on Ser473 (Delcommenne *et al.*, 1998; Lynch *et al.*, 1999; Persad *et al.*, 2000). Surprisingly, ILKAP inhibition of FN-induced ILK1 activity in the wt/6-4 and wt/6-10 clones did not affect FN-induced phosphorylation of PKB Ser473. Induction of the H154D mutant also had no inhibitory effect on Ser473 phosphorylation (Figure 5B). Inhibition of ILK1 kinase activity in these experiments requires catalytically active ILKAP.

In light of the unexpected Ser473 results in ILK1-inhibited cultures, it was important to determine the signalling outcome of ILKAP inhibition. ILK1-mediated phosphorylation of GSK3β has suggested a mechanism for the regulation of β-catenin nuclear translocation and activation of β-catenin–Lef transcription complexes, observed in ILK1-overexpressing epithelial cells (Novak *et al.*, 1998). We therefore examined whether ILKAP affected ILK1-stimulated phosphorylation of GSK3β, using phosphospecific antibodies to GSK3 Ser9 and Ser21. Transfectant clones were serum starved prior to stimulation with FN or insulin-like growth factor (IGF-1). As expected, these treatments induced robust phosphorylation on Ser9. Muristerone-induced ILKAP inhibited both FN- and IGF-1-induced Ser9 phosphorylation in the wt/6-4 and wt/6-10 clones (Figure 6), consistent with the reported effect of ILK1-stimulated Ser9 phosphorylation of GSK3β. We verified that phosphorylation of PKB on Ser473 was normal in the same wt/6-4 lysates (Figure 6A). To examine the requirement for ILKAP catalytic activity in the observed inhibition, we examined Ser9 phosphorylation in a stable cell line conditionally expressing the H154D catalytic mutant. Muristerone induction of the H154D line mutant did not inhibit FN-induced Ser9 phosphorylation (Figure 6B), indicating that ILKAP catalytic activity is required for inhibition of GSK3β Ser9 phosphorylation.

Overexpression of $p59^{ILK1}$ and ILK1 activity has been shown to induce nuclear translocation of β-catenin–Lef complexes and activation of a Tcf-responsive promoter. We reasoned that inhibition or blocking of Ser9 phosphorylation by overexpression of $p47^{ILKAP}$ activity would result in inhibition of growth factor-stimulated β-catenin–Lef activity. We chose to assay inhibition of β-catenin–Lef transactivation using the TOPFlash/FOPFlash reporter genes, which are widely used for this purpose. To test the requirement for ILKAP catalytic activity, we also assayed the H154D mutant for effects on TOPFlash reporter gene activation. The ILKAP-related PP2Cα has been reported to activate this pathway (Strovel *et al.*, 2000), thus providing a stringent specificity control for regulation of β-catenin–Lef transactivation. Three independently derived stable cell lines, expressing muristerone-inducible V5-PP2Cα, were used as controls for ILKAP activity. We confirmed muristerone-dependent expression of V5-tagged PP2Cα recombinant protein and PP2C activity in these PP2Cα transfectant lines (not shown). Serum stimulated high levels of luciferase activity from the TOPFlash reporter, as expected. This β-catenin-

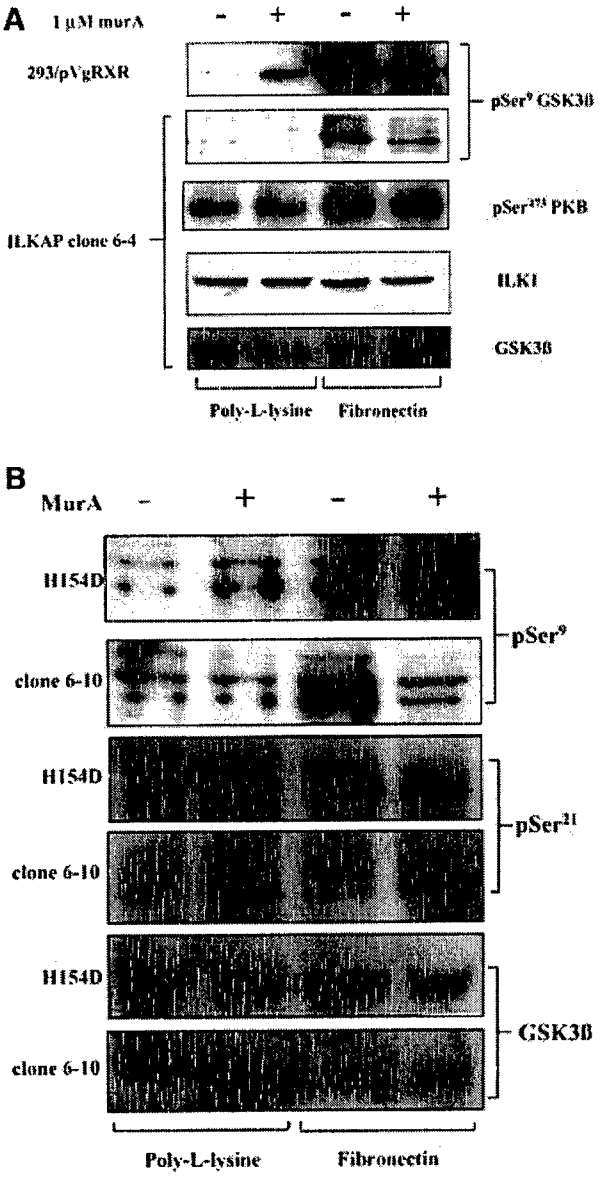

Fig. 6. ILKAP selectively inhibits phosphorylation of GSK3β at Ser9. (**A**) ILKAP clone wt/6-4 and vector control transfectant cells were serum starved and pre-treated or left untreated, prior to plating on FN. Control cells showed FN-dependent induction of Ser9 phosphorylation, which was inhibited in the wt/6-4 cells induced with muristerone A. Parallel blots were probed with phosphospecific antibody for PKB Ser473. Induction of ILKAP had no effect on Ser473 phosphorylation. Blots were stripped and reprobed with appropriate antibodies, for total ILK1 and GSK3 protein levels. (**B**) ILKAP clone wt/6-10 was treated as in (A), in parallel with the non-clonal transfectant cell line expressing a catalytically inactive H154D ILKAP mutant. Muristerone induction of ILKAP suppressed FN-induced Ser9 phosphorylation; however, the catalytic mutant did not inhibit this phosphorylation. Parallel blots were probed for changes in Ser 21 of GSK3α/β, which was not detectably affected by ILKAP activity. Blots were stripped and reprobed for total GSKβ protein levels.

dependent Tcf transcription factor activity was dramatically (50–70%) inhibited by muristerone induction of ILKAP, whereas induction of H154D mutant or PP2Cα did not inhibit β-catenin–Lef activity. Next we tested the ILKAP-mediated inhibition of β-catenin–Lef transactivation in response to insulin and IGF-1, conditions shown above to strongly induce GSK3β Ser9 phosphorylation in the 293 cells. Insulin- or IGF1-stimulated transactivation was inhibited by 60–75% in muristerone-treated wt/6-10 cells compared with levels in non-muristerone treated wt/6-10 and vector control cells, consistent with the inhibition of GSK3β Ser9 phosphorylation under these conditions (Figure 7B). These results indicate that ILKAP catalytic activity selectively inhibits β-catenin–Lef transactivation.

It was important to confirm that ILKAP inhibition of β-catenin–Lef transactivation is not generalized for other cytoplasmically activated transcription pathways. Type I interferon (IFN) activation of ISRE-dependent transcription requires cytoplasmic S/T (p38 MAP kinase) and tyrosine kinase (Tyk2 and JAK) activities for phosphorylation of STAT factors, and PI3K activity is also stimulated by IFN treatment of cells. We transfected ISRE-luciferase and TOPFlash reporter plasmids into the ILKAP clones, wt/6-4 and wt/6-10, as well as the H154D mutant and PP2Cα cell lines. Induction of ILKAP, H154D or PP2Cα did not inhibit IFN-stimulated ISRE reporter gene activity (Figure 7A). Taken together, the reporter assays are consistent with the data on inhibition of GSK3β phosphorylation, and indicate that ILKAP selectively targets GSK3β-regulated β-catenin–Lef transactivation.

Discussion

As demonstrated by sequence similarity and biochemical characterization of its catalytic function, ILKAP is clearly a PP2C. The PP2C family is a structurally diverse group of Mg^{2+}/Mn^{2+}-dependent S/T phosphatases, members of which have been implicated in environmental stress responses in yeasts, plants and mammals, as well as regulating abscisic acid signal transduction in *Arabidopsis thaliana* (Rodriguez, 1998). Rat PP2C, which shares 95% amino acid identity with ILKAP, was cloned using an amplification strategy to isolate conserved PP2C isoforms (Tong *et al.*, 1998). This orthologue is the most closely related protein to ILKAP that we have identified through searches of SwissProt and GenPept databases. The biochemical distinction of ILKAP and rat PP2C from other PP2C enzymes, i.e. being inhibited rather than stimulated by Mg^{2+}, further suggests that they are homologues and, thus, PP2C/ILKAP regulation of ILK signal transduction is likely to be conserved among mammals.

The crystal structure of PP2C has been resolved to 2 Å (Das *et al.*, 1996). This structure, along with extensive mutational analyses of mouse PP2Cβ (Kusuda *et al.*, 1998) and the related AB11 and AtPP2C phosphatases of *Arabidopsis* sp. (Sheen, 1998), have identified highly conserved PP2C residues that are critical for catalysis. Our site-directed mutations of ILKAP targeted the putative ^{152}DGH^{154} catalytic site, as analogous mutations in the *Arabidopsis* and mouse PP2Cβ enzymes have been shown to abrogate catalytic activity. Three ILKAP mutations, D152A, H154L and H154D, were markedly catalytically deficient, losing >90% of activity in *in vitro* phosphatase

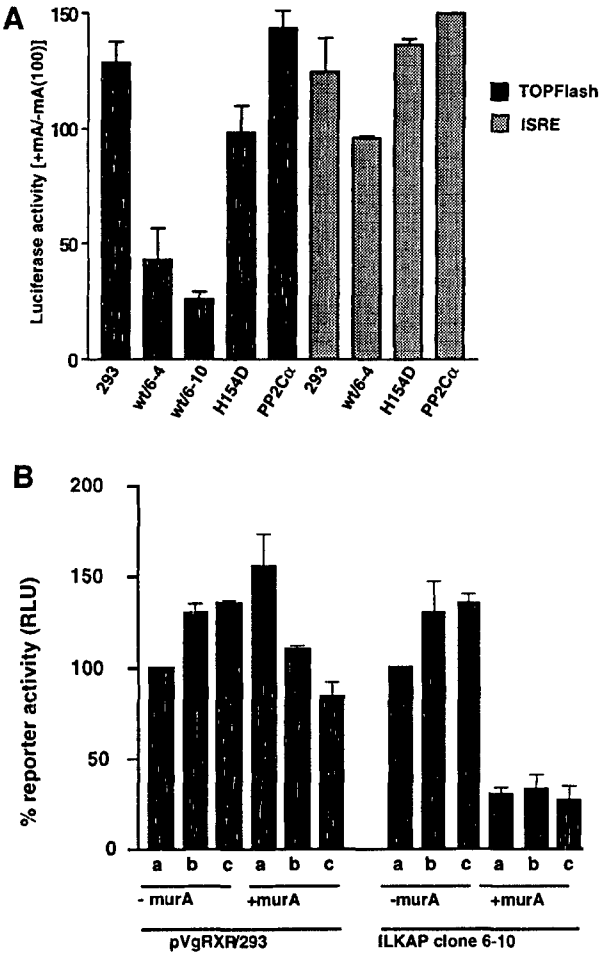

Fig. 7. Tcf/Lef factor activation is selectively inhibited by ILKAP. (A) TOPFlash and FOPFlash (mutant Tcf site) reporter plasmids were co-transfected with pRSV/βgal into 293/pVgRXR vector control, ILKAP wt/6-4 and wt/6-10, H154D and PP2Cα expressing lines (dark bars). Cells were untreated or muristerone A was added for 36 h to induce recombinant protein expression, and resulting Tcf-driven or ISRE-luc-driven luciferase reporter gene activity assayed. In the case of the ISRE-luc transfectants, recombinant human IFN-α was added (500 IU/ml) for the last 18 h of the induction period. Control experiments confirmed ~3-fold induction of ISRE-luc activity by this IFN treatment (data not shown). Luciferase activity is presented as the muristerone-induced/uninduced ratio, also corrected for luciferase activity from duplicate FOPFlash mutant reporter transfections in the case of FOPFlash results. (B) The Tcf-luciferase reporter construct TCF-pGL2 (see Materials and methods) was co-transfected with pRSV/βgal into the pVgRXR/293 vector control and ILKAP clone wt/6-10 cell lines. Transfectants cultured in 10% FCS (a), 10% FCS + 50 ng/ml IGF-1 (b) or 10% FCS + 100 nM insulin (c) were not induced or induced with muristerone A, and subsequently assayed for TCF-driven luciferase activity. Each transfection in (a), (b) and (c) was controlled internally and normalized to β-galactosidase activity, to account for transfection efficiencies.

assays (Figure 3B). These data indicate that the ^{152}DGH^{154} amino acid triplet does constitute the ILKAP active site, typical of PP2C. Each of the ILKAP catalytic mutants formed stable complexes with p59^{ILK1}; however, they did not inhibit FN-induced ILK1 activity, GSK3β Ser9 phosphorylation or β-catenin–Lef transactivation, indicating that they are not dominant-negative mutations. Our initial studies on ILK1 indicated that its constitutive overexpression in epithelial cells leads to oncogenic transformation, as assayed in soft agar colony and nude mouse tumorigenesis assays (Hannigan *et al.*, 1996; Radeva *et al.*, 1997). Constitutive expression from either antisense or dominant-negative ILK1 constructs has demonstrated that increased ILK1 activity is sufficient to effect transformation (Dedhar and Hannigan, 1996; Novak *et al.*, 1998; Wu *et al.*, 1998). We are currently testing the ILKAP catalytic mutants to determine whether they effect persistent, rather than transient ILK1 activation.

Our data support the view that p59^{ILK1} is a bona fide protein kinase, as we have previously demonstrated (Hannigan *et al.*, 1996), and suggest that ILKAP selectively inhibits ILK1 protein kinase activity and signalling. Recently, the catalytic subunit of PP2A has been reported to co-precipitate and co-localize with ILK1 and β1 integrin, in focal adhesions of F9 stem cells. The ILK1–integrin association in these cells is apparently regulated by PP2A-sensitive phosphorylation of β1 integrin, since ILK1 dissociates from phosphorylated β1 in OA-treated F9 cells (Mulrooney *et al.*, 2000). ILKAP activity is not OA sensitive, clearly distinguishing it from integrin-associated PP2A activity. The demonstrated selectivity of ILKAP in modulating ECM- or growth factor-induced phosphorylation of GSK3β Ser9, while having little or no effect on PKB Ser473 phosphorylation, suggests that ILKAP might directly dephosphorylate Ser9. However, our data on inhibition of ILK1 activity lead us to favour a mechanism whereby ILKAP inhibits ILK1-mediated Ser9 phosphorylation. We cannot rule out the possibility that GSK3β complexes with and inhibits ILK1 activity. *In vitro* studies using recombinant proteins should clarify these issues.

It is not known whether ILK1 activity is developmentally regulated by Wnt factors; however, recent evidence suggests differential regulation of β-catenin signalling by insulin and Wnt (Ding *et al.*, 2000). These authors show that Wnt, in contrast to insulin, does not induce phosphorylation of GSK3β or PKB, suggesting that the ILK1–GSK3β axis is not sensitive to Wnt. GSK3β activity phosphorylates β-catenin and targets it for destruction via the ubiquitin–proteasome pathway (Aberle *et al.*, 1997; Easwaran *et al.*, 1999; Barker *et al.*, 2000). ILK1-mediated inhibition of GSK3β induces nuclear translocation of stabilized β-catenin–Lef-1 complexes and activation of Tcf transcription factors (Novak *et al.*, 1998). In contrast to the effects of ILKAP on Tcf activity, we show that PP2Cα does not inhibit transactivation (Figure 7), and indeed PP2Cα has recently been shown to stimulate β-catenin–Lef signalling (Strovel *et al.*, 2000). Pharmacological inhibition of ILK1 activity results in reduced phosphorylation of GSK3β on Ser9 in SW480 colon carcinoma cells (Tan *et al.*, 2001), consistent with results presented here indicating the modulation of ECM- and insulin/IGF-induced GSK3β phosphorylation by the activities of ILK1 and ILKAP. Our data suggest further that ILK1/ILKAP function coordinately to regulate PKB-independent signalling of GSK3β, and clearly point to distinct mechanisms regulating ILK1 signalling of these protein kinases. Finally, the question of ILKAP regulation by extracellular signals provides a focus

for studies on the mechanisms of activation of ILK1 signalling.

Materials and methods

cDNA library screens and construction of inducible expression vectors

Two libraries (human placenta and human muscle) from OriGene (OriGene, Rockville, MD) were screened using an external vector-specific 5′ primer (Ori-3, 5′-GCAGAGCTCGTTTAGTGAACC-3′) and an internal ILKAP primer spanning the 3′ stop codon (CJLH-3, 5′-TCA-GTGCCCTAT-3′). Nested PCR was performed with Ori-2 (5′-TGG-GCGGTAGGCGTGTACGG-3′) and 3′ gene-specific primers (CJLH-1, 5′-ATAACGACACAAGAT-3′; or CJLH-4, 5′-CCCGAGTAATGA-GGG-3′). Two full-length ILKAP clones were obtained from the human muscle library. A partial ILKAP clone, missing the 5′ end of the gene (encoding amino acid residues 269–392), was obtained from the human placental cDNA library.

OriGene library screening was as recommended by the manufacturer. Four microlitres of each well from the master plate were screened using the Qiagen *Taq* DNA polymerase kit. Reactions contained 10 ng of vector-specific primer Ori1, 10 ng of ILKAP-specific primers, 2 μl of 10× reaction buffer, 0.2 μl of *Taq*, 0.4 μl of 2 mM dNTP, 0.4 μl of 25 mM $MgCl_2$, 4 μl of 'Q' buffer and 4 μl of library DNA. Thermocycles were as follows: 94°C for 10 min; (94°C for 1.15 min denaturation, 65°C for 1.0 min annealing with 0.5°C touch down per cycle, 72°C for 2 min extension) × 20 cycles; (94°C for 1.5 min, 55°C for 1.0 min, 72°C for 2 min) × 10 cycles; and a final 72°C for 7 min final extension (PE Biosystems GeneAmp 9700). Screening of the OriGene subplates was as recommended by OriGene, in which 5 μl of bacterial cells were used as in the above PCR reactions. Positive wells from the subplates were plated out onto LB/ampicillin agar plates and colony hybridizations to an ILKAP-specific probe were used to identify positives. The complete ILKAP clones were sequenced using either Amersham's Thermo-sequenase kit or by automated sequencing.

A full-length ILKAP cDNA (H4C11) was used as a template for PCR cloning into the pIND/V5TOPO-His expression vector (Invitrogen) allowing for ecdysone-inducible expression of V5 epitope-tagged recombinant ILKAP. A primer at the 5′ UTR of ILKAP (5′-GGC-ACCAGGCCCGC-3′) and a 3′ primer in which the TGA stop codon was removed (5′-GTGCCCTATCCGCAC-3′) were used to amplify the complete ILKAP using the OriGene clones as templates and Stratagene's high-fidelity *Pfu* DNA polymerase. Two microlitres of PCR product were immediately ligated into the pIND/TOPOV5-His vector. Colonies recovered following transformation were screened for the presence of inserts using *Bam*HI and *Xba*I restriction digests. Positive clones were sequenced to verify the ILKAP insert and to check for in-frame expression between ILKAP and V5/His coding regions. A clone designated H4C11-30 was used for subsequent transfections. Control plasmids expressing human PP2Cα were generated via the same strategy, using gene-specific primers (forward: 5′-ATAATGGGAGCATTTTTA-GACAAG-3′; reverse: 5′-CCACATATCATCTGTTGATGTAGA-3′) to generate an amplification product from a pcDNA3.1-PP2Cα plasmid template (Invitrogen). Independent pIND/V5TOPO-His/PP2Cα plasmid isolates were used to derive three stable pooled transfectant lines from the 293 pVgRXR clone described above.

Cell culture, transfections and establishment of stable transfectants

HEK 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), high glucose (Ontario Cancer Institute, Toronto, Ontario, Canada) supplemented with 10% fetal calf serum, 2 mM L-glutamine and 0.1 mM non-essential amino acids (Life Technologies) at 5% CO_2 and 37°C. Transfected cells were selected in 125 μg/ml zeocin (Invitrogen, Carlsbad, CA) and/or 400 μg/ml Geneticin G418 (Life Technologies, Burlington, Canada). Cells in 35 mm dishes were transfected with 1 μg of plasmid DNA in the presence of 4 μl of Lipofectamine Plus reagent (Life Technologies) under serum-free conditions (Optimem; Life Technologies). Cells were cultured for 16 h at 37°C; Lipofectamine/Optimem was removed and replaced with regular 10% fetal calf serum (FCS)/DMEM. Muristerone A (Invitrogen) was added to transiently transfected cultures at a final concentration of 1 μM for 24–36 h for induction of transgene expression. A stable HEK 293 derivative was generated by transfection of the pVgRXR (Invitrogen) expression plasmid, encoding a modified ecdysone receptor. This line was cloned out by limiting dilution and used as a recipient line for transfection of pIND expression plasmids. Double transfectants were maintained under G418 and zeocin selections. For stable lines, transfectant cells were allowed to recover for 48 h, following which G418 and/or zeocin was added for selection. Killing curves on the parental 293 cells established the concentration of each drug used for selections. Twelve independent isolates of HEK 293 transfectant clones were analysed for muristerone A-inducible V5-ILKAP expression levels. Lines were chosen for further study based on intermediate levels, relative to constitutively expressing clones, of recombinant ILKAP protein expression. For study of mutant ILKAP expression, a stable, pooled transfectant line was established by transfecting a mutated (see below) pIND/V5-ILKAP(H154D) plasmid into 293/pVgRXR cells and selection in G418 and zeocin. Stable clonal and pooled, non-clonal lines of HEK 293 transfectants were analysed for expression levels of recombinant V5-tagged ILKAP by western blotting, using V5 monoclonal antibody. Independent clones were selected by two rounds of limiting dilutions in 96-well tissue culture plates.

Site-directed mutagenesis

Three mutants were created using the full-length pIND/V5-ILKAP cDNA (H4C11-30, above) as template. These mutations were created by converting Asp152 to alanine (D152A), His154 to leucine or to aspartic acid (H154L or H154D). The following primers were used. The 5′ primer for all reactions was C26: 5′-GGCCGGATCCGGCACCAGGCC-CGCTGCTGC. Reactions were 3′ reverse-primed by one of the following internal primers, incorporating the underlined point mutations: C27 (5′-CTCGAATTCCTCCATCTCCATC), C28 (5′-CTCGAATTCCTC-CTGTCCAGCAA) and C29 (5′-ACTCGAATTCCTCCAAGTCCATC) using the parental H4C11-30 clone as template. PCR was performed using 10 ng of 5′ primer C26 and either of the 3′ primers C27, C28 and C29, 2 μl of 10× reaction buffer, 0.2 μl of *Pfu* thermostable polymerase (high-fidelity *Taq*), 0.4 μl of 25 mM dNTP, 0.4 μl of 25 mM $MgCl_2$ and 4 μl of template. The amplification cycles were as follows: 94°C for 10 min; (94°C for 1.25 min denaturation, 65°C for 1.0 min annealing with 0.5°C touch down per cycle, 72°C for 2 min extension) × 20 cycles; (94°C for 1.5 min, 55°C for 1 min, 72°C for 2 min) × 10 cycles; and a final 72°C for 10 min extension. A 500 bp *Bam*HI–*Eco*RI fragment was cut out of the cDNA clone H4C11-30, and the *Bam*HI–*Eco*RI linkered mutant PCR product was directionally cloned in, to replace the wild-type fragment in the template vector. Positive clones were sequenced to verify the presence of the mutation and to check for in-frame fusion of ILKAP with the V5 epitope.

Fibronectin, IGF-1 stimulation and immune complex kinase assays

Sterile non-tissue culture dishes (100 mm) were coated overnight with 10 μg/ml FN (Life Technologies) or poly-L-lysine (Sigma, St Louis, MO) in phosphate-buffered saline (PBS) pH 7.4 at 4°C with gentle rotation. Prior to use, these plates were blocked for 2 h at 37°C with 2.5 mg/ml BSA (Sigma; Fraction V) in DMEM. Cells were harvested and washed twice in 2.5 mg/ml BSA/DMEM. A total of 2×10^6 cells were added to each plate in 5 ml volume and incubated for 20 min at 37°C. Non-adherent cells were removed and the plates were washed twice with ice-cold PBS. Cell lysis was performed directly on the plates in 200 μl of NP-40 lysis buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 50 mM HEPES pH 7.5, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 3 mM phenylmethylsulfonyl fluoride). Following 20 min incubation on ice, the soluble lysate was collected by centrifugation at 15 000 *g* for 20 min at 4°C. A non-adherent control was performed on 100 mm non-tissue culture plates pre-coated with BSA/DMEM. For IGF-1 (Sigma) and insulin stimulations, cells were pre-starved in serum-free medium overnight. Cells were treated with 50 ng/ml IGF-1 or 100 nM insulin for the specified times at 37°C, and washed twice with cold PBS followed by lysis at 4°C.

Kinase assays were carried out as described in Hannigan *et al.* (1996). Protein concentration was determined by Bradford assays (Bio-Rad, Richmond, CA). Cytoplasmic lysates (0.2–0.5 mg) were immunoprecipitated with 1.5 μg of affinity-purified rabbit anti-ILK [cat. #06-592; Upstate Biotechnology Inc. (UBI), Lake Placid, NY] overnight at 4°C, with rotation. Protein A–Sepharose (Sigma), pre-swollen in NP-40 lysis buffer, was added for 2 h at 4°C to capture the antibodies. Following two washes with NP-40 lysis buffer and two washes with kinase wash buffer [10 mM $MgCl_2$, 10 mM $MnCl_2$, 50 mM HEPES pH 7.5, 0.1 mM sodium orthovanadate, 1 mM dithiothreitol (DTT)], kinase assays were performed directly on the protein A beads. Kinase assay reaction was performed in 25 μl volume containing 10 mM $MgCl_2$, 10 mM $MnCl_2$, 50 mM HEPES pH 7.5, 1 mM sodium orthovanadate, 2 mM sodium

fluoride, 5 mCi [γ-^{32}P]ATP (Amersham, Piscataway, NJ) and 12.5 μg of MBP (UBI). Incubation was for 30 min at 30°C. The reaction was stopped with 10 μl of SDS–PAGE non-reducing stop buffer and heated for 5 min at 95°C. Phosphorylated MBP bands were visualized by 10% SDS–PAGE and autoradiography on Kodak X-OMAT film (Eastman Kodak, Rochester, NY) or by PhosphorImager analysis (Storm; Molecular Dynamics). Kinase assays to assess the activity of ILKAP on ILK1-mediated phosphorylation were carried out in the absence of sodium orthovanadate or sodium fluoride phosphatase inhibitors.

In vitro assay for S/T phosphatase activity

Phosphatase assay was carried out as outlined in the New England Biolabs instruction manual. The protein concentration of all samples was determined by Bradford assays (Bio-Rad). Protein (20–50 μg) was pre-incubated at 30°C for 2–5 min with assay buffer (50 mM Tris–HCl pH 7.0, 0.1 mM Na_2EDTA, 5 mM DTT, 0.01% v/v Brij 35) plus 5 μM OA (PP1, PP2A inhibitor) and 0.1 mM EGTA (PP2B inhibitor), 4 mM Mn^{2+} and 4 mM Mg^{2+} (when indicated). Reactions were started by adding 10 μl of [γ-^{32}P]ATP-labelled MBP and incubating for 10 min at 30°C. The reaction was then terminated by adding 200 μl of ice-cold 20% trichloroacetic acid (Sigma) and placing on ice for 5–10 min. Tubes were spun at 12 000 *g* for 5 min at 4°C. Finally, 200 μl of supernatant were added to 2 ml of aqueous scintillation fluid and counted.

Western blot analyses of protein expression

SDS–PAGE gels were transferred to polyvinyldifluoride membrane (Immobilon-P; Millipore, Bedford, MD) by electrophoresis in 25 mM Tris, 192 mM glycine and 20% methanol. For anti-ILK, anti-ILKAP, anti-V5 westerns, membranes were blocked in 5% milk in TBST (20 mM Tris pH 7.5, 500 mM NaCl, 0.1% Tween-20). Primary antibodies against ILK1 (UBI), V5 epitope (Invitrogen) and anti-ILKAP (affinity-purified rabbit polyclonal) were diluted to 1 μg/ml in TBS with 1% (w/v) BSA (Fraction V). Incubation was for 2 h at room temperature with gentle shaking. Secondary antibodies of either horseradish peroxidase (HRP)-conjugated goat anti-rabbit or rabbit anti-mouse (Jackson ImmunoResearch) were incubated at 1/20 000 dilutions in TBST, for 45 min at room temperature. Bands were visualized with chemiluminescent substrate (ECL; Amersham).

Phosphospecific antibodies, recognizing PKB Ser473 and Thr308 (New England Biolabs), as well as GSK-3 Ser9 (courtesy of Dr J.Woodgett, Ontario Cancer Institute, and BioSource, Camarillo, CA) and Ser21 (UBI), were used for analyses of the phosphorylation status of PKB and GSK3. A GSK3 antibody (clone 4G-1E; UBI) was used for determination of total GSK3α/β protein levels. Western blot protocols supplied by the manufacturer (NEB, Beverly, MA) were strictly adhered to. Primary antibodies were diluted in 0.1% Tween-20, 20 mM Tris pH 7.6 and 136 mM NaCl, and incubated overnight at 4°C with rotation. Secondary antibodies were used at 1/20 000 for 45 min at room temperature.

Immunoprecipitation

Cells transiently transfected with the H4C11-30 ILKAP (full-length) constructs were induced for 18 or 36 h with 1 μM muristerone A. Cells were harvested in NP-40 lysis buffer and protein concentrations determined by Bradford assay. Lysate (1 mg) was first pre-cleared with 100 μl of protein A (50% slurry equilibrated in NP-40 buffer) for 2 h at 4°C, then incubated with 1–2 μg of affinity-purified rabbit anti-ILK, or rabbit anti-ILKAP antibodies, overnight at 4°C. Protein A–Sepharose was added for an additional 2 h at 4°C, to capture primary immune complexes. Complexes were washed 8–10 times in NP-40 lysis buffer, samples run on 12% SDS–PAGE gels and transferred to Immobilon-P membranes. Westerns were performed with anti-V5^{HRP} (Invitrogen), affinity-purified anti-ILK (UBI) or affinity-purified anti-ILKAP antibodies.

Alternatively, immunoaffinity columns were prepared in which rabbit anti-ILK was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia, Montreal, Quebec, Canada). Lysates were applied to the immobilized protein beads and rotated overnight at 4°C. Following extensive washes with 10× volume lysis buffers, the bound material was eluted with 100 mM glycine pH 3.0 and/or 100 mM triethanolamine pH 11.5, and immediately neutralized with 1 M Tris pH 8.0. The eluted material was run on SDS–PAGE, transferred to PVDF membranes and blotted with appropriate antibodies.

Luciferase reporter gene assays

TOPFlash and FOPFlash reporter plasmids (UBI) (1 μg/35 mm dish) were individually co-transfected with 1 μg of pRSV β-gal into stable ILKAP transfectant and vector control lines, to assay Tcf/Lef factor activation. As a second test of Tcf activation, cells were transiently transfected with 1 μg of TCF-pGL2, containing five tandem repeats of the TCF response element (Kinetek, Vancouver, BC, Canada) and 1 μg of pRSV-β-gal with Lipofectamine Plus reagent. For induction of recombinant ILKAP and mutant proteins, muristerone A was added for 36–48 h post-transfection. After the induction period, cells were washed twice with PBS and harvested in 100 μl of Promega reporter lysis buffer (Promega, Madison, WI). Twenty microlitres of lysates were mixed with 100 μl of Luciferase Assay Reagents (Promega) and read immediately in a Berthold Lumat LB9501 luminometer. β-gal assays were performed according to Sambrook *et al.* (1989). Briefly, 20 μl of lysates were mixed with 3 μl of 0.1 M $MgCl_2$, 4.5 M β-mercaptoethanol, 66 μl of *o*-nitrophenyl-β-D-galactopyranoside and 0.1 M sodium phosphate pH 7.5. Incubation was for 30 min at 37°C. Colorimetric optical density was read at 420 nm in an ELISA reader (Molecular Devices, SpectroMax 250). Results are expressed as luciferase units (× 10^6)/β-gal values.

DDBJ/EMBL/GenBank accession No.

The DDBJ/EMBL/GenBank accession No. for ILKAP cDNA is AY024365.

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Integrin-linked Kinase-1, ILK1, is required for L6 myoblast differentiation

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ABSTRACT

Myogenic concentrations of insulin, or plating cells on extracellular matrix proteins, transiently induced ILK1 kinase activity in L6 myoblasts. Overexpression of the integrin-linked kinase (ILK1) in L6 resulted in increased ILK1 kinase activity, and increased formation of myotubes, as well as induction of myogenin and [Ca^{++}]ATPase differentiation markers. Expression of a catalytically inactive ILK1 mutant, ILK(E359K), blocked the induction of endogenous ILK1 activity, morphologic differentiation and myogenic gene expression by insulin. Cell cycle analysis of ILK(E359K) cells cultured in serum-free conditions indicated a significant degree of apoptosis (11-19% sub-diploid peak) which was abrogated by insulin, IGF-1, or 10% serum. Expression of ILK variants did not have profound effects on S-phase transit, however. Insulin-stimulated phosphorylation of PKB at Ser473 was unimpaired in the ILK(E359K) cells, suggesting that PKB is not a myogenic target of ILK1, and that PKB-mediated survival of L6 myoblasts does not require ILK1. In contrast, lithium, an inhibitor of GSK3β, also blocked L6 differentiation and myogenic gene expression, suggesting the GSK3 arm of ILK1 signalling is important in L6 differentiation.

INTRODUCTION

Primary myogenesis is a tightly regulated process involving the fusion of committed myoblasts to form multinucleate myotubes, with parallel induction of muscle-specific regulatory genes. In vitro modelling of this early myogenic step, using the rat L6 and murine C2C12 myoblast lines (Yaffe, 1968), has been useful in establishing the role of muscle regulatory factors such as MyoD, Myf5 and myogenin (MRFs) in determining myoblast fate. More recently, work in these systems has begun to identify intracellular pathways linking extracellular myogenic signals, such as those provided by insulin, insulin like growth factors (IGFs) and the extracellular matrix (ECM), to changes in expression and activity of MRFs. Phosphatidylinositol 3 kinase (PI3K) is particularly important in myoblast differentiation, since inhibition of PI3K activity by the pharmacologic agents wortmannin or LY294002, or via expression of a dominant negative p85 PI3K subunit, blocks IGF1-induced L6 myoblast differentiation (Jiang et al., 1998, Chang et al., 1997, Kaliman et al., 1998). Moreover, IGF-II induction of L6 myoblast differentiation requires PI3K-dependent activation of NFκ-B transcription factor (Kaliman et al., 1996), indicating the involvement of non-muscle specific nuclear factors in myogenic signalling. The serine/threonine kinase, PKB, is a well-documented target of PI3K signalling which is particularly important in mediating cell survival. In this light, it has been shown that inhibition of PKB activity by a dominant negative mutant blocks fusion of chick embryo myoblasts (Jiang et al., 1999), suggesting that PKB mediated survival is critical for CEM differentiation. These studies point to the importance of the PI3K-PKB axis in signalling myogenesis.

ILK1 (integrin-linked kinase) is a cytoplasmic protein serine/threonine kinase which is an important target of PI3K-mediated integrin signal transduction (Hannigan et al., 1996, Dedhar et

al., 1999). The $p59^{ILK1}$ protein complexes with ß1 integrins and localizes with integrins to focal adhesion plaques (Tu et al., 1999, Li et al., 1999), further supporting its role in regulating cell responses to ECM. Growth factors such as insulin, or ECM induce a rapid, transient increase in ILK1 activity, and experiments employing dominant negative and pharmacologic inhibitors of PI3K have indicated that ILK activation by these disparate receptors is dependent on PI3K activity (Delcommenne et al., 1998). Moreover, loss of the PTEN tumour suppressor gene has recently been shown to elevate ILK activity levels, in turn activating PKB, indicating that gain of PI3K activity stimulates ILK-mediated signalling (Morimoto et al., 2000, Persad et al., 2000). Overexpression of ILK1 in mammary epithelial cells results in mesenchymal transformation of these cells, suggesting a morphogenetic role for ILK1-mediated signals in epithelial cells (Somasiri et al., 2001). *Drosophila* ILK mutants are embryonic lethal, displaying defects in muscle attachment and integrin-mediated adhesion (Zervas et al., 2001). The role of ILK in *Drosophila* muscle development, coupled with the fact that ILK is a target of PI3K, prompted us to investigate the potential role for ILK signal transduction in vertebrate myogenesis.

In this study, we demonstrate that ILK activity is elevated during insulin-induced L6 myoblast differentiation, and that this elevation is blocked by pre-treatment with wortmannin and LY294002, inhibitors of PI3K activity. Stable L6 transfectants expressing a kinase-deficient variant of ILK, ILK(E359K), which is shown to act in a dominant negative fashion, are unable to differentiate under conditions of serum withdrawal, or treatment with insulin or IGF1. In contrast, cultures of L6 cells expressing an active ILK transgene display increased numbers of morphologically and biochemically differentiated cells relative to control myoblast cultures, which exhibit a very low background of myotube formation. The ILK dominant negative mutant

completely blocked induction of myoblast differentiation, however, insulin-stimulated survival and PKB activity were unimpaired, indicating a specific role for ILK in L6 differentiation. Taken together, our data indicate that ILK1 is a critical effector of PI3K myogenic signalling.

RESULTS

ILK1 kinase activity is modulated in L6 cells by extracellular matrix and insulin

Laminin (LN), merosin (laminin-2, MN) and fibronectin (FN) have all been implicated in regulating aspects of myogenesis in vitro and in vivo. To investigate the potential role of ILK1 in myogenic signalling, we tested the ability of these ECM proteins to stimulate ILK1 kinase activity in the L6 myoblasts. ILK1 kinase activity was robustly stimulated by each ECM protein (Fig. 1). However, we did observe differences in activation kinetics in each case. ILK1 was stimulated on FN at 5 and 30 minutes, while LN induced a rapid transient activation, which was maximal at 5 minutes. Conversely, MN induction of ILK1 activity displayed relatively delayed kinetics, with maximal activation at 30 minutes. These results confirm that ILK1 activity is stimulated in L6 cells interacting with ECM proteins known to play a role in myogenic differentiation and survival.

Insulin is a potent differentiating agent for myoblasts, and has been shown to activate ILK1 in epithelial cells, thus, we examined ILK1 activity in insulin-treated L6 cells. ILK1 activity was stimulated at 10 minutes post-insulin treatment in both myoblasts and differentiated myotubes, with activity decreasing by 20 minutes (Fig. 2a). The response in both cell populations was similar, and pre-treatment with the PI3K inhibitors, wortmannin or LY294002, essentially blocked these responses. Insulin-induced ILK1 activation was blocked in stable cell

lines transfected with a kinase-deficient ILK1 construct, ILK1(E359K). Treatment of ILK(E359K) cells (four independent clones) with insulin failed to stimulate ILK1 kinase activity, although vector control transfectants showed normal, LY294002-sensitive ILK1 induction (Fig. 2b). Similarly, this ILK mutant protein acts as a dominant negative suppressor of ILK1 signalling in epithelial cells. Induction of differentiation did not affect ILK1 protein expression levels, as judged by western blotting of myoblast and myotube lysates (not shown). Our results indicate that activation of ILK1, by myogenic concentrations of insulin or by ECM, is dependent on PI3-K activity, consistent with previous findings in epithelial cells.

Expression of a dominant negative ILK1 mutant blocks induction of L6 differentiation-

We next sought to address the role of ILK1 in myoblast differentiation. Stable L6 transfectants carrying expression vectors encoding full length active (ILK-wt1), or kinase-deficient (dominant negative) mutant ILK1(E359K) proteins were analyzed for ILK1 effects on myoblast differentiation. The ILK(E359K) cells were unable to fuse and form myotubes under conditions that normally induce differentiation (Fig. 3a). Control transfectants showed normal levels of myotube formation when cultured in DM, with a minimal background of myotube formation in GM, however under growth (ie. non-differentiating) conditions ILK1 wild-type transfectants exhibited more extensive fusion and myotube formation than did the control cultures (Fig. 3a). We conclude from the morphological experiments that ILK1 kinase activity is required for L6 myoblast fusion and formation of multinucleated myotubes.

In order to to determine whether the ILK(E359K) effect on L6 cell morphology reflected a block to cell fusion, or represented failure to initiate biochemical differentiation, we assayed transfectant clones for induction of the muscle-specific genes, myogenin and

[Ca^{+2}]ATPase. Myogenin is a marker of myoblast differentiation, and in L6 myoblasts induced in DM, myogenin expression is also induced and peaks at 48 hours after induction. Myogenin levels were equivalent or slightly elevated in the ILK-wt transfectant cells, compared to undifferentiated controls. However, expression of myogenin was completely abolished in ILK(E359K) transfected cells (Fig. 3b), consistent with the observed block to morphological differentiation in these cells. The levels of sarcoplasmic [Ca^{+2}]ATPase were assayed in membrane-enriched preparations from the L6 transfectant clones. As was the case with myotube induction and myogenin expression, control myoblasts upregulated [Ca^{+2}]ATPase expression upon differentiation. However, L6 expressing ILK(E359K) showed a complete block to [Ca^{+2}]ATPase expression under growth or differentiation conditions. Consistent with this, ILK-wt1 transfectants showed a dramatic induction of [Ca^{+2}]ATPase, to above control levels in both the myotube and myoblast populations. The bulk of cellular of $p59^{ILK1}$ was localized to the membrane fraction, consistent with its association with integrin subunits, and these data confirmed elevated levels of ILK1 protein in the ILK-wt1 expressing cells, relative to control transfectants (Figure 4). These results demonstrate that transfection of ILK1 is both necessary and sufficient to induce the L6 differentiation program.

As cell cycle withdrawal is a prerequisite for myoblast differentiation, we examined the effect of the ILK-wt and ILK(E359K) transgenes on L6 cell cycle profile. L6 ILK-wt1 and ILK(E359K) transfectant cultures stained with propidium iodide and analysed by FACS, showed expression of these transgenes does not affect the L6 cell cycle profile under GM or DM culture conditions (Table I). Under serum-free conditions, unlike cells cultured in serum, ILK(E359K) transfectant cultures exhibited a significant (11%-19%) apoptotic fraction, which was not

observed under equivalent conditions for control or ILK-wt transfectants. Insulin treatment of the ILK(E359K) protected against apoptosis (Table I), indicating that insulin provides a survival signal in the absence of detectable ILK activation. Similar protective effects were seen with IGF1 treatment, which also induces a rapid transient stimulation of ILK1 activity in L6 control cultures (data not shown). The protective effect of insulin on the ILK(E359K) cells indicates that expression of the dominant negative ILK1 protein does not impair insulin receptor function. Western blotting of transfectant cell lysates also indicated equivalent levels of insulin receptor expression among ILK-wt1, ILK(E359K) and control L6 cultures (data not shown). L6 cells undergo insulin-induced formation of membrane-localized bundles of actin filaments known as membrane ruffles (Khayat et al., 2000). The ILK-wt1, ILK(E359K), and vector control L6 cells all form typical membrane ruffles with insulin stimulus (not shown). These results indicate that ILK1-mediated differentiation is not a consequence of cell cycle withdrawal, and that the block to differentiation in ILK(E359K) cells results from loss of ILK1 signalling.

ILK1 has been implicated in the activation of PKB (Delcommene et al, 1998, Lynch et al., 1999), which itself has been shown to provide critical survival and differentiation signals during myoblast differentiation (Jiang et al., 1999, Fujio et al., 1999). Full activation of PKB requires phosphorylations on both Thr308 and Ser473, mediated by PDK1 and PDK2 activities, respectively. ILK1 has been shown to mediate growth factor- and integrin-stimulated PDK2 activity in epithelial cell lineages, and may represent a PDK2. We thus examined insulin stimulation of PKB Ser473 phosphorylation in ILK-wt and ILK(E359K) L6 cultures. Surprisingly, we found that insulin stimulated Ser473 phosphorylation equivalently in the ILK-wt- and ILK(E359K)-expressing lines (Fig. 5a). Thus, inhibition of ILK1 activity by the dominant

negative mutant does not significantly inhibit PKB activation, indicating that PKB activation by insulin is not sufficient for L6 myoblast differentiation.

ILK1 activity has been shown to inhibit signalling by GSK3β, which regulates the β-catenin transcriptional co-activator by phosphorylating and targetting it for proteolytic degradation. LiCl is known to inhibit GSK3β activity, thus, we examined the effect of LiCl treatment on both ILK1 activity and myoblast differentiation. Pretreatment of the parental L6 cells with 25mM LiCl blocked induction of myogenin expression by insulin, (Figure 6a) but did not inhibit insulin activation of ILK1 (Figure 6b). The Li-treated cells also failed to form myotubes, correlating with the block to myogenin induction (data not shown). These results indicate that ILK1 activation is not sufficient for differentiation, and further suggest a Li-sensitive target of ILK1 in the myogenic pathway. It is likely that one Li-sensitive ILK1 target is GSK3β, however we observed no differentiation-associated alteration in β-catenin expression levels (Fig 5b) or phosphorylation (data not shown). These data suggest that both ILK1 and GSK3β are required, but inhibition of GSK3β by ILK1 is not sufficient, for myoblast differentiation.

DISCUSSION

We have shown here that L6 myoblast differentiation to multinucleated myotubes requires the activity of ILK1. Previous work has demonstrated that ILK1 stimulates/supports S phase transit, anchorage independent growth, and survival of epithelial cells (Hannigan et al., 1996, Radeva et al., 1997, Wu et al., 1998). Unlike many dominant acting oncogenes, ILK1 overexpression induces serum-dependent colony growth in soft agar, suggesting that receptor tyrosine kinases signal through ILK1 to stimulate integrin-independent growth. Indeed, it has

been shown in a variety of epithelial cells, that growth factors such as insulin transiently stimulate ILK1 protein kinase activity in synergy with integrin-mediated adhesion (Delcommene et al., 1998, Persad et al., 2000). Here we have shown that ILK1 is stimulated by insulin/IGFs and ECM proteins in myoblasts, and further that stimulation of ILK1 by either route is blocked by pretreatment of cells with the PI3K inhibitors, wortmannin and LY294002. PI3K activation may involve interaction of PIP_3 with ILK1, since this lipid has been shown to activate ILK1 kinase activity in vitro (Delcommenne et al., 1998). Although there is a potential YxxM PI3K interaction motif in the N-terminal region of ILK1, it seems unlikely that this motif functions in mediating the direct association of PI3K with ILK1, since ILK1 is not detectably tyrosine phosphorylated after insulin treatment of L6 cells (MM and GH, unpublished data). Our results suggest that committed myoblasts require PI3K activation of ILK1 for fusion and terminal differentiation.

Recently, PDGF and IGF1 have been shown to activate distinct survival pathways in mouse C2C12 myoblasts. PDGF-induced survival was shown to mediated through the ERK pathway, while IGF1-mediated survival resulted from sustained PI3K dependent phosphorylation and activation of PKB (Lawlor et al., 2000). In C2C12 cells transfection of an ILK1 expression construct has been shown to increase ERK expression levels, presumably preventing differentiation by this mechanism (Huang et al., 2000). However, transgene-dependent changes in ILK1 activity were not confirmed in the C2C12 study. Our results in L6 myoblasts clearly indicate that ILK1 activity promotes, and inhibition of ILK1 activity suppresses differentiation. We do not understand the molecular basis of the differential ILK effects on C2C12 and L6 myoblast differentiation, however this may reflect an influence of cell context on

ILK1 function. The L6 model affords us the opportunity to study the role of ILK1 protein interactions in myogenesis, and a comparative study of these interactions in the contexts of both C2C12 and L6 differentiation would serve to clarify this issue.

ILK1 integration of distinct signal inputs from receptor tyrosine kinases and integrins raises the question of whether these inputs reflect distinct functions in the L6 model. Ectopic expression of ILK-wt1 or ILK(E359K) in the L6 cells does not have a significant effect on cell growth or cycling, thus the observed block to myoblast differentiation in the ILK(E359K) cells reflects a bona fide differentiation effect, and is not simply the consequence of cell cycle withdrawal. As well, the ILK(E359K) cell cultures do exhibit a significant apoptotic index (11%-19%) under serum free conditions, indicating that ILK1 signaling is required for some aspects of myoblast survival. The insulin-stimulated phosphorylation of PKB at Ser473 is robust in the ILK(E359K) cells, and this activation is likely to play a role in the protective effect of insulin in these cells. Thus, coordinated activation of ILK1 and PKB in response to myogenic signals may be required to complete the differentiation program, however in the absence of ILK1 activity, PKB is still capable of transducing protective signals. Indeed, ectopic expression of a dominant-negative PKB was found to increase, and wild type PKB decrease the apoptotic index in differentiating C2C12 myoblasts (Fujio et al., 1999), indicating PKB is a positive mediator of myoblast survival, consistent with our results in L6 myoblasts.

GSK3β is a component of the Wnt developmental signal transduction pathway, and is also an important target of ILK1 signalling. Inhibition of GSK3β activity by pretreatment of L6 myoblasts with LiCl blocked both, myogenin expression (Fig. 6) and myotube formation (not shown), suggesting the GSK3β is required for ILK1-mediated differentiation. Interestingly, LiCl

has recently been shown to mimic Wnt signalling in developing quail muscle (Borycki et al., 2000). Our data suggest that both ILK1 and GSK3β activities are required for L6 differentiation. Importantly, LiCl did not inhibit insulin induction of ILK1 activity (Fig. 6), indicating that inhibition of GSK3β activity is not the key mechanism of ILK1 myogenic signalling. We are currently examining the L6 lines for effects of ILK expression and activation on β-catenin nuclear localization, as well as the possibility that ILK signals target transcriptional regulation of myogenin, and other MRFs.

MATERIALS AND METHODS

Reagents- Media for cell culture were purchased from the media preparation facility of the Ontario Cancer Institute (Toronto). Fetal bovine serum (FBS), fibronectin (FN), merosin (MN), laminin (LN), Lipofectamine, antibiotic/antimycotic, G418, 0.25% trypsin/EDTA and insulin (bovine) were obtained from Life Technologies (Burlington, Ontario, Canada). Polyclonal anti-ILK1 antibody and myelin basic protein were purchased from Upstate Biotechnologies Inc. (UBI, Lake Placid, New York), or alternatively MBP was a generous gift of Dr. Mario Moscarello (Hospital for Sick Children, Toronto, Ontario, Canada). Anti-insulin receptor antibody (Ab-4) and LY294002 were purchased from Calbiochem (San Diego, California, USA). Anti-phosphotyrosine PY20 and anti-PKB/Akt antibodies were from Transduction Labs, and anti-phosphoserine473 antibody from New England Biolabs. The F5D anti-myogenin monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank, University of Iowa. [γ^{32}P]ATP was from PharMingen (Mississauga, Ontario, Canada). Protein

A-Sepharose beads, wortmannin, bovine serum albumin (BSA), and monoclonal anti-β actin antibody (AC-15) were purchased from Sigma (Oakville, Ontario, Canada). We obtained PVDF Immobilon P transfer membrane from Millipore (Mississauga, Ontario). Autoradiography and chemiluminescence results were imaged on Kodak X-Omat film. All tissue culture plastics (NUNC) were purchased from Life Technologies (Burlington, Ontario, Canada).

cDNA Vectors, Transfection, and Cell Culture- L6 rat skeletal myoblasts (obtained from Dr. A.Klip) were grown in α-MEM supplemented with 10% FBS and 1% antibiotic/antimycotic (GM). For differentiation, cells were grown to 80% confluency and then media was replaced with α-MEM supplemented with 2% FBS and 1% antibiotic/antimycotic (DM) and allowed to differentiate for 2-6 days, as indicated. cDNAs encoding a full length active, and a kinase-deficient dominant negative ILK(E359K) cDNA, was subcloned into pcDNA3.1 (Invitrogen, Carlsbad, California, USA). Expression vectors were transfected into second passage L6 cells using Lipofectamine, and transfected cells were selected with 850 μg/mL (determined to be lowest completely lethal concentration with parental L6) G418. Stable clones were isolated by limiting dilution, and only passages between 3 and 10 used for experiments. To assay insulin stimulation of ILK1 activity cells were cultured under serum-free conditions (α-MEM with antibiotic/antimycotic) overnight. To inhibit PI3K activity, cells were pre-treated for 20 min with 200 nM wortmannin, or 20 μM LY294002, in serum-free medium. Cells were treated with 100 nM insulin in serum-free medium for indicated times, and then lysed *in situ* in NP40 buffer for kinase assay as described above. To assay ECM stimulus of ILK1 activity, L6 cells were seeded into 6-well tissue culture dishes coated with fibronectin (FN), laminin (LN), merosin

(MN) (all at 10 μg/ml) or bovine serum albumin (BSA) at 2.5 mg/ml as a negative control. Cells were then lysed *in situ* at indicated time points, and assayed for ILK1 kinase activity as described below.

Nuclear Staining of L6 - To visualize nuclei in L6, cells grown on glass coverslips were fixed with 70% ethanol and stained with hematoxylin. Excess stain was washed off with double distilled water, and cells were visualized and photographed at 40x magnification under a light microscope.

Preparation of Total Membranes- Total L6 membranes were prepared as previously described (Sargeant et al., 1993). Briefly, cells were rinsed twice with cold homogenization buffer (250 mM sucrose, 20 mM HEPES pH 7.4, 5 mM NaN_3, and 2 mM EGTA). Cell monolayers were scraped into cold homogenization buffer containing 1uM leupeptin, 1 uM pepstatin, 10 uM E-64, and 200 uM phenylmethylsulfonyl fluoride (PMSF), homogenized with 20 strokes (Dounce type A homogenizer), and then centrifuged at 700g for 5 min at 4C. The supernatant from this low-speed spin was then centrifuged for 1h at 190,000g to obtain the total membrane fraction used for western blot analyses.

Western blotting- To assay protein expression levels, we harvested and lysed L6 cells in Nonidet P-40 (NP40) lysis buffer (150mM NaCl, 1% NP-40 (v/v), 0.5% sodium deoxycholate (w/v), 50mM HEPES pH 7.5, 1 μg/mL each aprotinin and leupeptin, 50 μg/mL phenlmethylsulphonyl fluoride), or alternatively, isolated total membrane fraction as shown above. Protein concentration was measured by the Bradford method using the BioRad protein assay kit. 100 μg NP40 lysate was separated on 12% SDS-PAGE gels and transferred to PVDF

membrane (80V, 1.25hr). PVDF membranes were blocked in 5% non-fat milk overnight, and with primary antibody at recommended dilutions (α-ILK1 0.5μg/mL, F5D 1:100, A52 1:500, AB-4 1:400, α-PKB 1:500, AC–15 1:5000, α-ser473 1:1000)

ILK1 Immune Complex Kinase Assays- For analysis of ILK1 kinase activity, cells were lysed in NP40 buffer, supplemented with 1mM sodium orthovanadate and 5 mM sodium fluoride as phosphatase inhibitors. Equal amounts of protein from these cell lysates were immunoprecipitated with α-ILK1 polyclonal antibody as previously described (Hannigan et al., 1996), and immune complexes were incubated at 30^0C for 30 min with myelin basic protein (2.5 μg/reaction) and γ[^{32}P]ATP (5 μCi/reaction). The reactions were stopped by addition of 4X concentrated SDS-PAGE sample buffer (Laemmli, 1970). Phosphorylated proteins were separated on 15% SDS-PAGE gels. MBP bands were visualized by autoradiography with X-Omat film. As a control for equal ILK1 among assayed samples, immune-complexes were electrophoresed, transferred to PVDF membrane and probed with anti-ILK1 antibody as described above.

FACS Analysis- L6 cells were harvested with trypsin/EDTA, and equal numbers of cells were washed with PBS/2% FCS and fixed in 70% ethanol on ice for 30 minutes. Cells were washed in PBS containing 2% FCS (v/v), then in 70% ethanol, and stained with 0.1 mg/ml propidium iodide/0.6% NP40 (v/v) with 2mg/mL RNAse in the dark, at room temperature for 30 minutes. Cell suspensions were filtered through 85um Nitex mesh and kept at 4^0C in the dark, until ready for FACS analysis. For cell cycle analyses, nuclei were stained with propidium iodide, and data from 15000 single cells based on doublet discrimination were acquired by a FACSCAN analyzer (BD Biosciences, San Jose, CA) and fitted using the DNA analysis program Modfit LT 2.0 (Verity

Software, Maine).

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Cells	Vector control				ILK(E359K)				ILK-WT			
Conditions	GM	DM	SF	INS	GM	DM	SF	INS	GM	DM	SF	INS
Subdiploid	0.2 0.04	0.08 nd	2.7 4.0	0.01 0.02	0.06 0	0.06 nd	19.2 11.1	2.8 0.32	0.05 0.0	0.0 nd	0.05 0.29	0.01 0.40
S phase	23.3 23.3	19.1 nd	13.3 17.7	24.4 32.5	26.4 32.9	22.6 nd	8.7 13.8	18.6 21.7	24.3 36.1	19.2 nd	9.4 19.9	23.1 38.0
G2-M	11.5 8.9	9.8 nd	7.6 6.3	10.5 9.6	14.2 20.8	12.4 nd	7.7 5.5	12.2 13.1	10.8 14.9	9.5 nd	6.3 7.5	8.7 12.3
Go-G1	65.1 67.8	71.1 nd	79.1 76.0	65.2 57.9	59.4 46.3	64.9 nd	83.6 80.5	69.2 65.2	64.9 49.0	71.3 nd	84.3 72.7	68.2 49.6

Table I: Cell cycle analysis of L6 myoblasts (vector control and ILK transfectants) under growth (GM), differentiating (DM), serum-free (SF), and serum-free plus 100nM insulin (INS). Duplicate numbers are from two independent experiments. Nd, not done.

FIGURE LEGENDS

Figure 1. ILK1 kinase activity is stimulated in L6 myoblasts by ECM proteins relevant to myogenesis. L6 myoblasts were plated on fibronectin (FN), laminin (LN), or merosin (MN) (10 μg/ml), or BSA control (2.5 mg/ml) for 5 and 30 minutes. ILK1 kinase activity was measured by in vitro ILK1 immune complex kinase assay using myelin basic protein (MBP) as exogenous substrate. These results are representative of at least three independent experiments. Coomassie Blue staining of the gel indicated equivalent amounts of MBP present in each lane.

Figure 2. Insulin regulation of ILK1 activity in L6 myoblasts **(a)** Insulin-stimulated L6 myoblasts (10% FCS) and myotubes (2%FCS, 6 days) were assayed for ILK1 catalytic activity in immune complex kinase assays, using MBP as exogenous substrate. Cells were treated for the indicated times with 100 nM insulin with or without 20 minute pre-treatment with the PI3-K inhibitor wortmannin. **(b)** Kinase-deficient, dominant-negative ILK1 mutant (E359K) blocks insulin-induced ILK1 activation. L6 myoblasts (ILK(E359K), and vector control) were assayed for ILK1 catalytic activity by immune complex kinase assay on MBP as exogenous substrate. Cells were treated for the indicated times with 100 nM insulin with or without pretreatment with the PI3-K inhibitor, LY294002 (20μM). These results are representative of four independent clones of each L6 transfectant.

Figure 3. Dominant negative ILK1 mutant blocks morphological and biochemical differentiation of L6 myoblasts. **(a)** Stable transfectant clones expressing ILK-wt1, ILK(E359K), or vector control were cultured in 10% (GM) or 2% (DM) serum conditions. Four independent clones of each (one representative clone of each is shown: vector control, ILK(E359K), ILK-wt1) were allowed to differentiate as described, fixed, stained for nuclei with hematoxylin, and photographed (40x). No significant myotube formation was seen in ILK(E359K) transfected clones, and myotube formation above background was seen under growth conditions in ILK-wt1 transfected clones. **(b)** Vector control and ILK(E359K) transfected L6 cells were grown for 48h after reaching 80% confluence under growth (GM-10%FCS) or differentiation (DM-2%FCS) conditions. At 48h, cells were lysed and assayed for myogenin expression by western blot. Membranes were stripped and re-probed with ILK antibody to control for levels of ILK1. Results were repeated three times in separate experiments.

Figure 4. Sarcoplasmic reticulum [Ca^{++}]ATPase induction upon differentiation of L6 myoblasts is completely eliminated by the ILK(E359K) variant, and is amplified by transfection with ILK-wt1. ILK-wt1 transfection results in increased ILK1 in membrane preps under differentiating conditions. Vector control, ILK(E359K) and ILK-wt1 transfected L6 cells were differentiated in 2% FCS for 6 days as described, and total membrane were analysed by western blot for sarcoplasmic [Ca^{++}]ATPase expression. β-actin blots show equal amounts of lysates used for membrane isolations (C^P: cytosolic, pre-centrifugation). Membrane (M) and cytosolic (C)

preparations were blotted for expression levels of $p59^{ILK1}$. Results shown are representative of two independent experiments.

Figure 5. **(a)** Dominant negative ILK(E359K) does not block insulin-induced Ser473 phosphorylation of PKB. Control (C) and insulin-induced (I) cultures of L6 expressing ILK(E359K), or ILK-wt1 were lysed and assayed by western blotting for levels of Ser473 (phospho-PKB), as well as total PKB and ILK1 proteins. Results are representative of three independent experiments. **(b)** ILK-wt transfection stimulates and ILK(E359K) blocks, insulin-induced myogenin expression. Vector control, ILK-wt, and ILK(E359K) cultures under growth (GM) or differentiation (DM) conditions, were treated with insulin and assayed for induction of myogenin by western blotting. Membranes were sequentially stripped and re-probed with antibodiy to ILK1, and β-catenin. Results are representative of three independent experiments.

Figure 6. (a) LiCl treatment inhibits myogenin induction in L6. L6 myoblasts were treated with 25 mM LiCl prior to imposition of growth (GM) or differentiation (DM) conditions. Cells were lysed and assayed for myogenin induction by western blotting. LiCl blocked myogenin induction effectively after 48 and 72 hr of differentiation induction. LiCl treated cells did not form myotubes (not shown). Membranes were stripped and reprobed for ILK1 levels (the last lane represents a loading artefact in this one experiment). **(b)** LiCl does not inhibit ILK1 activity. L6 transfectants were treated with 100 nM insulin for 10 min, with and without a 20 min pretreatment with 25 mM LiCl. ILK1 activity was assayed in immune complex kinase assays using exogenous MBP as substrate.

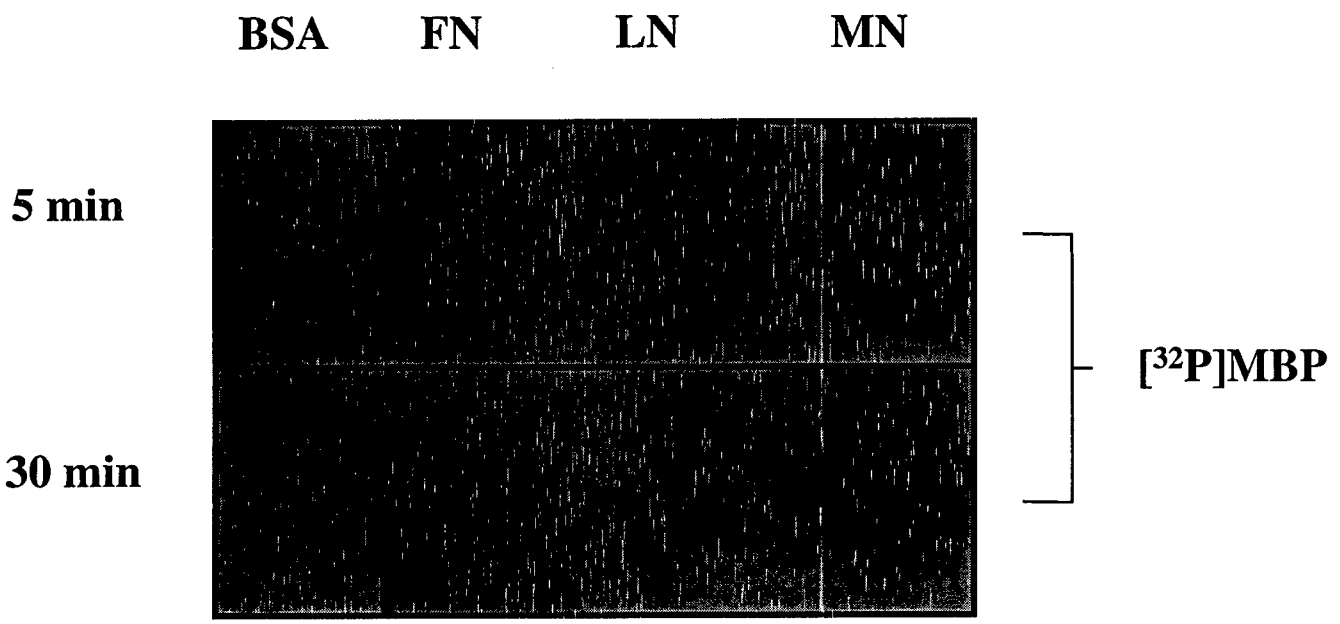
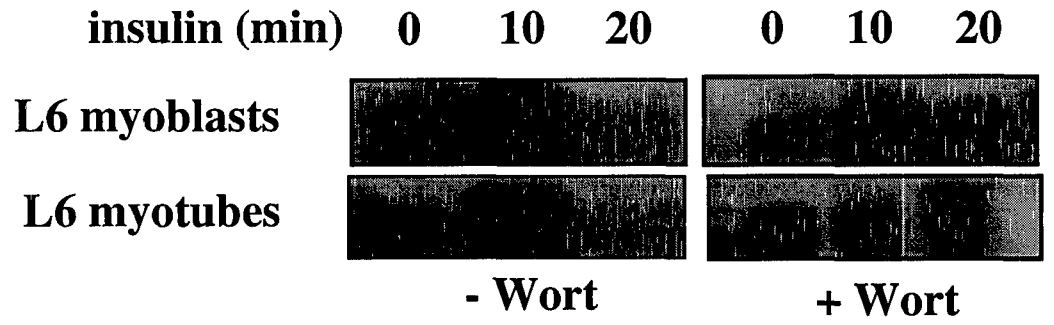


Figure 1

(a)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L6 myoblasts
L6 myotubes
- Wort
+ Wort

(b)

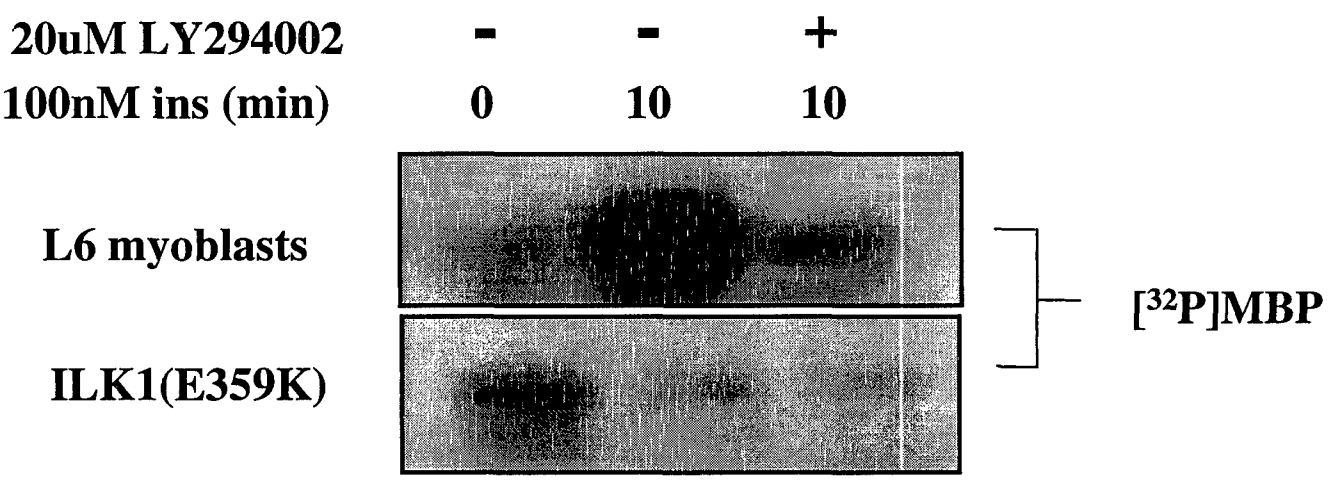
20uM LY294002 - - +
100nM ins (min) 0 10 10
L6 myoblasts
ILK1(E359K)
[32P]MBP

Figure 2

A.

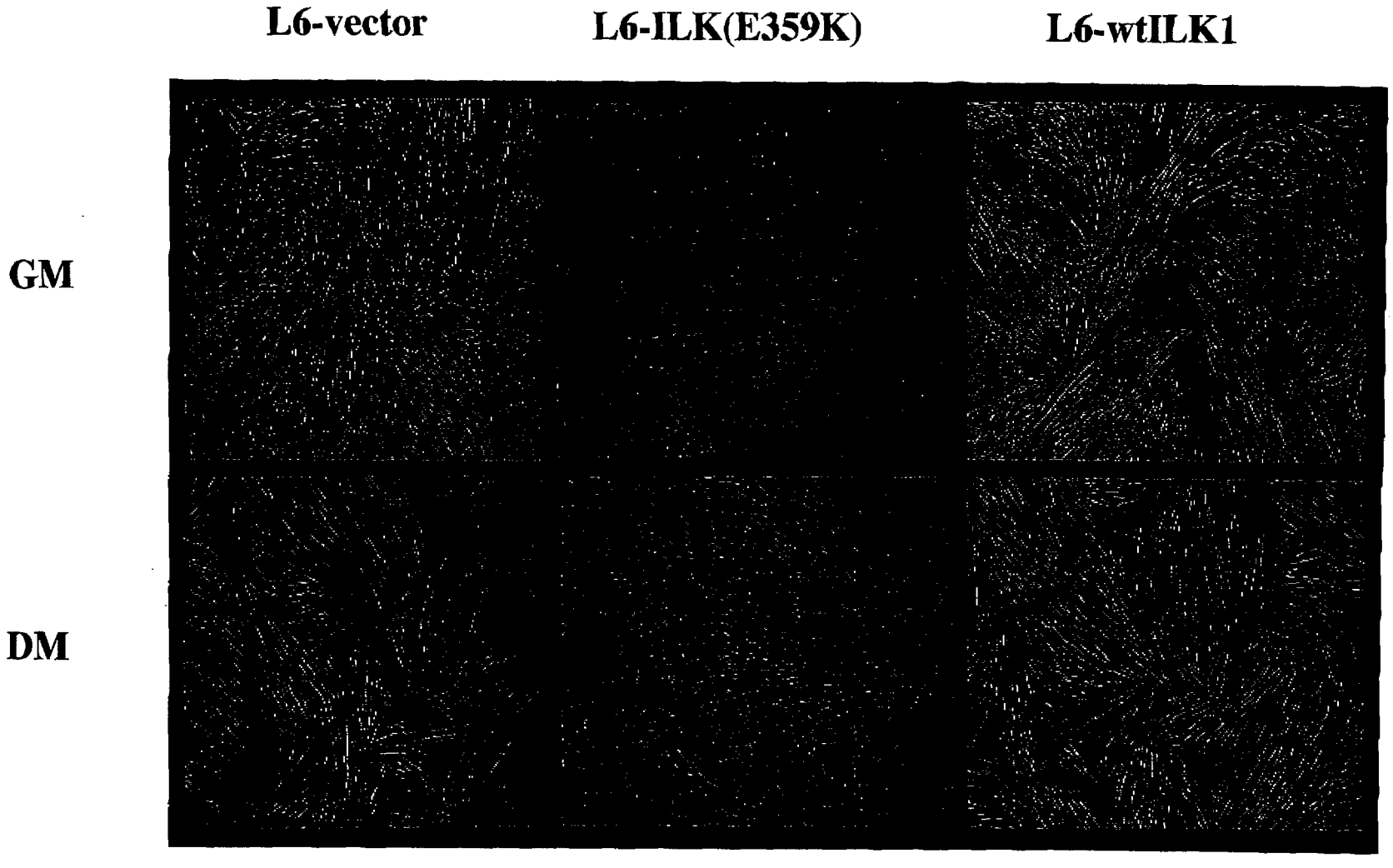

Figure 3.1, Miller et. al. (HPP.II)
DAMD17-97-1-7092

B.

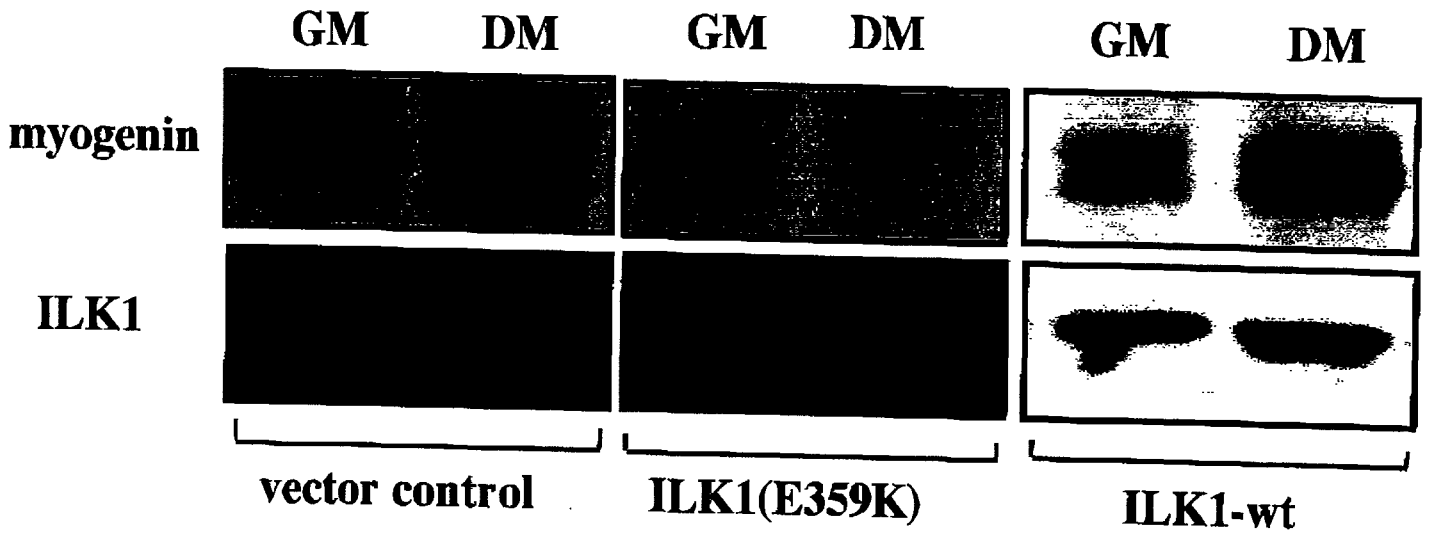

Figure 3B, Miller et. al. (APP.II)
DAMD17-97-1-7092

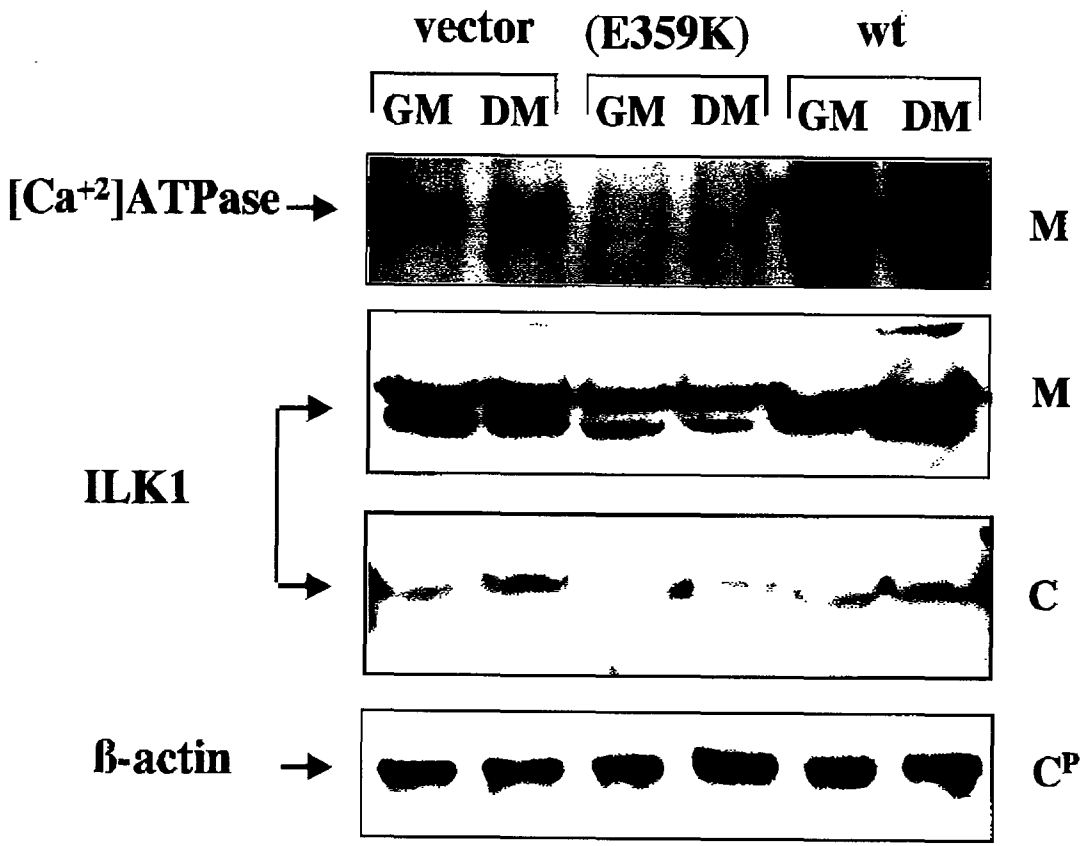

Figure 4. Miller et al. (Appendix II, DAMD17-79-1-7092)

A.

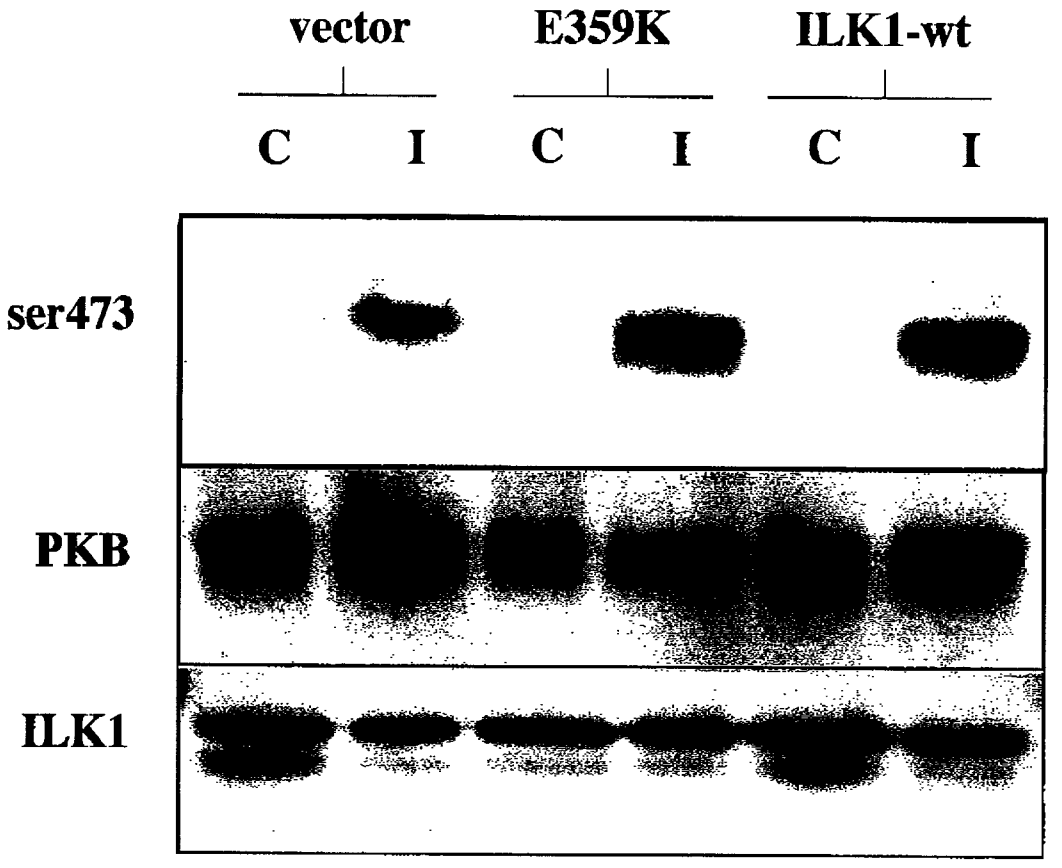

Figure 5A: Miller et al. (App. II, DAMD17-79-1-7092)

B.

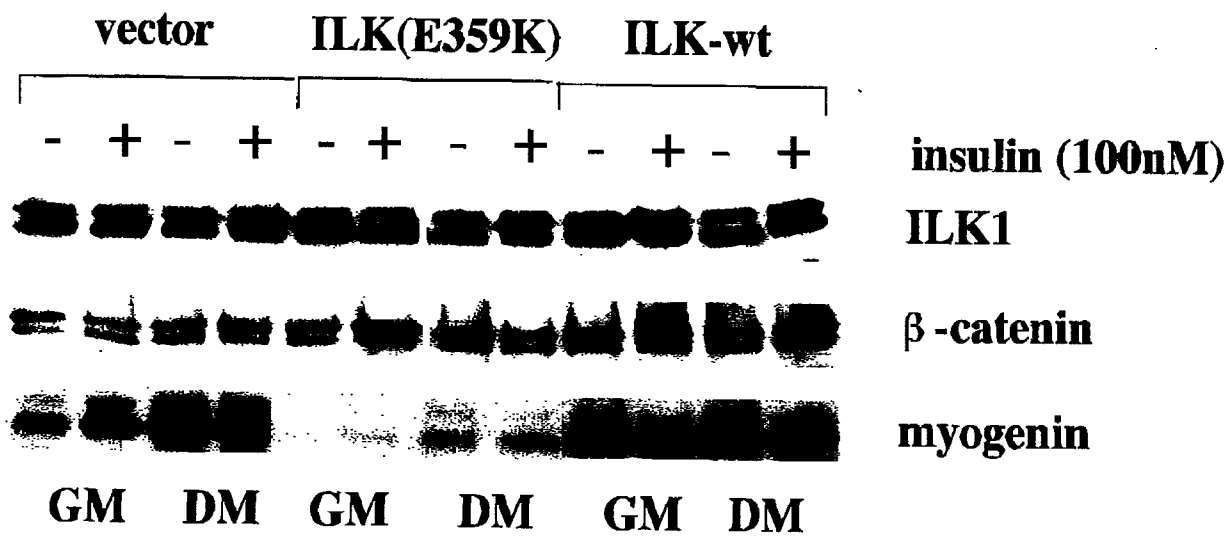

Figure 5B: Miller et. al. (App. II, DAMD17-97-1-7092)

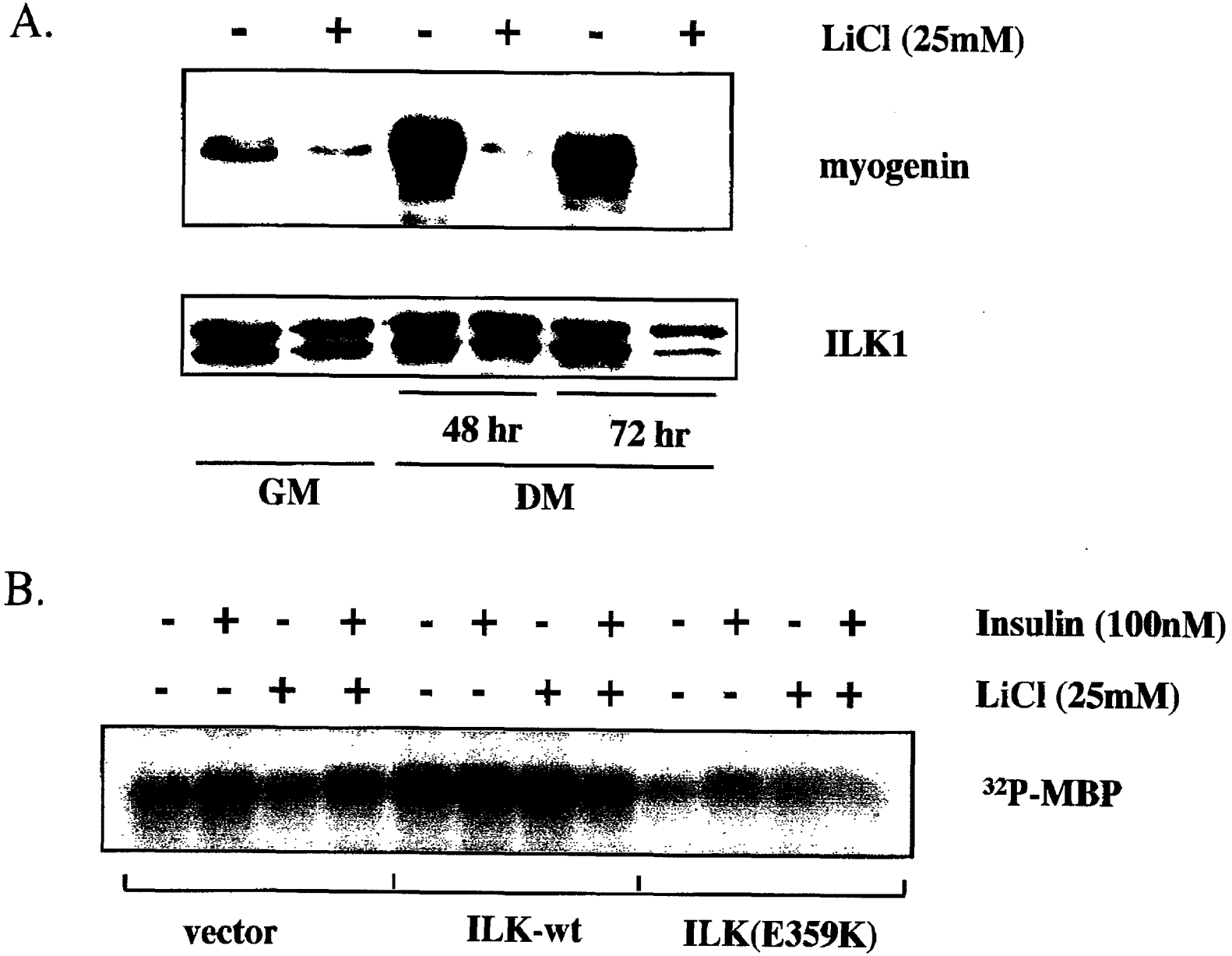

Figure 6: Miller et. al. (App. II, DAMD17-97-1-7092)